Oxidative Stress Resistance in *Drosophila melanogaster*

Thesis

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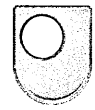

The Open University

Oxidative Stress Resistance in *Drosophila melanogaster*

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A thesis submitted in partial satisfaction for the degree of Doctor of Philosophy

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DECLARATION

The work contained in this thesis is entirely my own, and is the result of my own academic and experimental enquiry. Contributions to the work by colleagues are fully acknowledged in the text. This work has not been, and is not currently being submitted for candidature for any other degree.

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I'm not sure if it's all worth it in the end but it's been one hell of a ride.

ABSTRACT

As a result of normal physiological processes, organisms generate reactive oxygen species (ROS), which include hydrogen peroxide (H_2O_2) and some free radicals, including superoxide radicals ($O_2^{\bullet -}$). When the balance between pro- and antioxidants is perturbed, an organism is subjected to oxidative stress. This gives rise to oxidative damage, the accumulation of which has been implicated in ageing. This thesis describes work using *Drosophila melanogaster* as a model system to elucidate the relationship between oxidative stress and lifespan, focusing on glutamate-cysteine ligase (GCL), the rate-limiting enzyme in the glutathione pathway, a major component of the body's antioxidant defence mechanism.

GCL is a dimer, consisting of a ~23kDa modifier subunit (GCLM) with no individual catalytic activity and a ~73kDa catalytic subunit (GCLC). Under conditions of oxidative stress, the catalytic subunit, which has some individual activity, binds to GCLM to form a more catalytically efficient holoenzyme via the formation of disulphide bridges. Using the GAL4-UAS system, this study examined the consequences of perturbation of glutathione titres in relation to whole-organism physiology, over-expressing both sub-units (individually and in combination) in a variety of tissues. Results demonstrate that pan-neural over-expression can be beneficial, leading to an increase in lifespan. However, ubiquitous over-expression has been found to have serious detrimental effects during development. No correlation was demonstrated between the extended longevity phenotype and an increase in organismal oxidative stress resistance. These findings strongly suggest

that the relationship between a reduction in oxidants and the benefits to an organism is not a simple one. They also implicate neural tissue as a key lifespan-limiting tissue type.

PUBLICATIONS

Conference Abstracts

Kotecki C., Saunders R.D.C. (2009) Positive and negative effects on lifespan of viability of perturbation of glutathione titres in *Drosophila melanogaster.* 50th Annual *Drosophila* research conference, Chicago

Kotecki C., Akhtar R.A., Fraser J.A., Bone J., McLellan L.I., Saunders R.D.C. (2008) Positive and negative effects on lifespan of viability of perturbation of glutathione titres in *Drosophila melanogaster.* Cellular Senescence Workshop, Oriel College, Oxford, UK

Gilfillan J.C., Kotecki C., Kansagra P., Saunders R.D.C. (2004) Glutathione, oxidative stress and aging in *Drosophila..* Gordon Research Conference Biology of Aging meeting, Aussois, France

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ABBREVIATIONS

ALS	Amyotrophic lateral sclerosis
AP-1	Activator protein-1
ARE	Antioxidant response element
ATP	Adenosine triphosphate
BER	Base excision repair
cDNA	Complementary DNA
CR	Caloric restriction
DEM	Diethyl maleate
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
EGF	Embryonic growth factor
EpRE	Electrophilic response element
ETC	Electron transport chain
FALS	Familial amyotrophic lateral sclerosis
GCL	Glutamate cysteine ligase
GCLC	GCL catalytic subunit
GCLM	GCL modifier subunit
GFP	Green fluorescent protein
GI	Genito-urinary
GR	Glutathione reductase

GS	Glutathione synthetase
GSH	Glutathione
GSSG	Glutathione disulphide (oxidised GSH)
GST	Glutathione S-transferase
GPx	Glutathione peroxidase
Insulin/IGF	Insulin/Insulin-like growth factor
JNK	c-Jun N-terminal protein kinase
MHC	Myosin heavy chain
MMR	Mismatch repair
Msr	Methionine sulphoxide reductase
MTH1	mut T homologue 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NER	Nucleotide excision repair
NLS	Nuclear localisation signal
Nrf-2	Nuclear factor related factor 2
ROS	Reactive oxygen species
SOD	Superoxide dismutase
Trx	Thioredoxin
TrxR	Thioredoxin reductase
UAS	Upstream activating sequence

1. INTRODUCTION

Organisms generate reactive oxygen species as by-products of aerobic metabolism. Endogenous antioxidant defences, whilst being robust are imperfect and this results in an imbalance between pro- and antioxidants and it has been proposed that this leads to cumulative damage throughout the lifespan of an organism, ultimately resulting in cellular senescence and organismal death. Externally delivered and endogenously synthesised antioxidants have been identified as molecules that may have an impact on lifespan and healthspan in humans and are the focus of a body of research examining how enhancement of oxidative stress resistance affects lifespan in a variety of model organisms. -glutamylcysteinylglycine, the tripeptide commonly known as glutathione (GSH) is a key component of the organismal oxidative defence system and is one of the most abundant non-enzymatic antioxidants and the most important low molecular weight thiols as a result of its protective role. GSH biosynthesis is a two-step process catalysed by the enzymes glutamate-cysteine ligase (GCL) and glutathione synthetase (GS). This thesis focuses on the enzyme GCL, the rate-limiting enzyme in this pathway, as a candidate gene for manipulations that may ultimately lead to increased organismal damage protection and hence altered longevity. This chapter presents an overview of the causes of organismal oxidative stress and the cellular responses to it. Evidence for the involvement of the glutathione biosynthesis pathway in this response is reviewed alongside a review of current theories as to why organisms age, including the role of oxidative stress in the ageing process. Finally, *Drosophila melanogaster* is evaluated for its suitability as a model organism for ageing research.

1.1. Reactive Oxygen Species and Oxidative Stress

As a result of normal physiological processes, organisms generate reactive oxygen species (ROS), which can prove harmful. ROS is a collective term used to describe a group which includes hydrogen peroxide (H_2O_2) and some free radicals as detailed in Table 1.1, including superoxide radicals.

Table 1.1: Reactive Oxygen Species (Halliwell and Gutteridge 2007)

Radicals		Non-Radicals	
Superoxide	$O_2^{\bullet -}$	Hydrogen peroxide	H_2O_2
Hydroperoxyl	$HO_2^{\bullet}$	Peroxynitrite	$ONOO^-$
Hydroxyl	$OH^{\bullet}$	Peroxynitrous acid	ONOOH
Peroxyl	$RO_2^{\bullet}$	Nitrosoperoxycarbonate	$ONOOCO_2^-$
Alkoxyl	$RO^{\bullet}$	Hypochlorous acid	HOCl
Carbonate	$CO_3^{\bullet -}$	Hypobromous acid	HOBr
Carbon dioxide	$CO_2^{\bullet -}$	Ozone	O_3
Singlet Oxygen	$O_2^1 g^+$	Singlet Oxygen	$^1 g$

A free radical is defined as "any species capable of independent existence .... that contains one or more unpaired electrons" (Halliwell and Gutteridge 2007). It is designated by a superscript dot after the chemical formula. The ability of free radicals to exist in an energetically unstable form with unpaired electrons in their outer shell means they have the potential to be biologically destructive molecules. They are paramagnetic (i.e. weakly attracted to magnetic fields) and therefore combine readily with charged molecules (e.g. nucleic acids, proteins, lipids) and this can lead to oxidative damage to those molecules (Halliwell and Gutteridge 2007). The three main ROS generated by reduction of univalent oxygen are $O_2^{\bullet -}$, $OH^{\bullet}$ and H_2O_2 (Hulbert, Pamplona et al. 2007). Of these, $OH^{\bullet}$ is highly reactive and its generation through the combination of $O_2^{\bullet -}$ and H_2O_2 during the Fenton reaction is one reason for the significance of H_2O_2 as a source of oxidative stress (Hulbert, Pamplona et al. 2007). H_2O_2 is not classified as a free radical, lacking unpaired

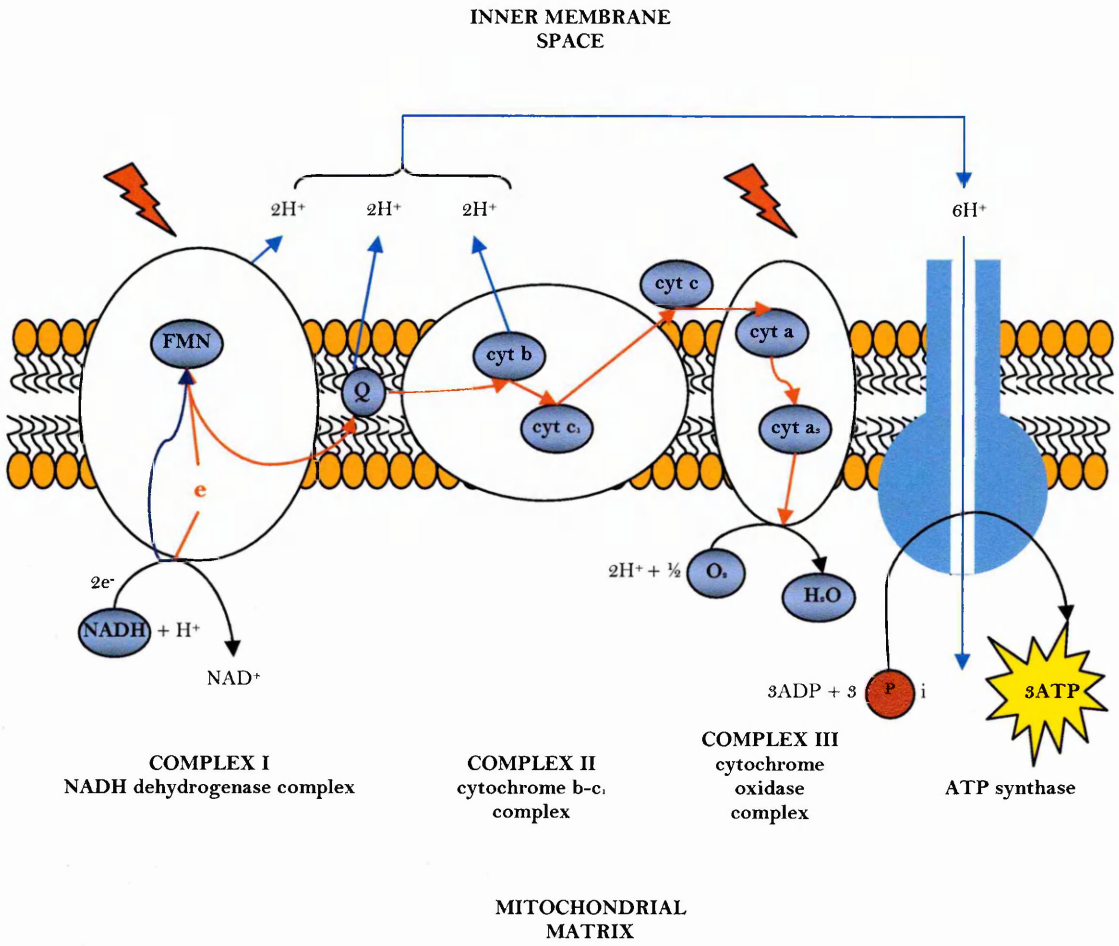
electrons, but is nonetheless very damaging. One of the most harmful aspects of H_2O_2 is its highly diffusive nature, which leads to the propagation of oxidative damage via the generation of $OH^{\bullet}$ and other reactive radicals far from the original source of insult despite the fact that it is a weak oxidising and reducing agent in itself (Halliwell and Gutteridge 2007; Hulbert, Pamplona et al. 2007).

1.1.1 Sources of ROS

Mitochondrial Electron Transport Chain

One of the major endogenous sources of ROS in an organism is thought to be the mitochondria (Sanz, Pamplona et al. 2006). During aerobic respiration, electrons are transferred along the mitochondrial electron transport chain (ETC) in a process that leads to the eventual generation of ATP, as shown in Figure 1.1. As a result of electron leakage in the early part of the electron transport chain (ETC) at Complex I (Barja and Herrero 1998; Herrero and Barja 2000) and Complex III during oxidative metabolism, superoxide ($O_2^{\bullet -}$) is generated (Boveris and Cadenas 2000; Miwa, St-Pierre et al. 2003). This is believed to be one of the most significant sources of ROS, with most cellular H_2O_2, an end product of the dismutation of $O_2^{\bullet -}$, originating from the mitochondria (Chance, Sies et al. 1979; Beckman and Ames 1998).

Figure 1.1: The components of the electron transport chain. The movement of the electron is indicated by the pink arrow. Movement of the electron is indicated by the red arrow. Proton pumps are indicated by blue arrows. FMN = flavoprotein, Q = ubiquiquinone, cyt = cytochome (Becker, Kleinsmith et al. 2000; Alberts, Johnson et al. 2008). Red lightning bolts represent points at which oxidative stress is generated due to electron leakage.



Superoxide dismutase (SOD) catalyses the dismutation of $O_2^{\bullet -}$ to hydrogen peroxide and oxygen (a simultaneous oxidation and reduction reaction), as shown in the following equation:

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

This reaction is catalysed by MnSOD in the mitochondria and by Cu/ZnSOD in the cytosol -these enzymes will be discussed in more detail in Section 1.4. It has not been

possible to measure free $O_2^{\bullet -}$ in intact mitochondria due to the presence of MnSOD (Beckman and Ames 1998) therefore most work has been done using isolated submitochondrial particles with SOD removed by sonication. The sonication process also removed cytochrome C, another rapid scavenger of $O_2^{\bullet -}$ *in vivo* (Forman and Azzi 1997). It is only when the ETC has been treated with inhibitors such as antimycin A that $O_2^{\bullet -}$ generation has been detected (Forman and Kennedy 1974; Loschen, Azzi et al. 1974). This raises questions about the existence of free $O_2^{\bullet -}$ within the mitochondria *in vivo*. Whilst MnSOD should act to increase the rate of $O_2^{\bullet -}$ generation *in vivo* by accelerating product removal by dismutation to H_2O_2, in reality, it is possible that its actual role is to increase H_2O_2 generation, with $O_2^{\bullet -}$ as a rapidly consumed intermediate (Forman and Azzi 1997; Beckman and Ames 1998). In recent years, the degree to which *in vitro* work accurately represents the situation *in vivo* has been called into question (Beckman and Ames 1998). Experimental procedures *in vitro* did not accurately mimic *in vivo* conditions, with oxygen saturation 15% higher *in vitro* (Beckman and Ames 1998) and concentrations of substrates 10-fold higher *in vitro* (Imlay and Fridovich 1991; Hansford, Hogue et al. 1997; Beckman and Ames 1998). Nonetheless, it is generally accepted that the mitochondrial contribution to organismal oxidative stress is high, with an estimated 2-4% of total oxygen consumed during electron transport converted to ROS (Beyer 1990).

Peroxisomes

During the oxidation of -fatty acids in the peroxisome, flavoprotein dehydrogenases react with O_2 to give H_2O_2 (Beckman and Ames 1998; Halliwell and Gutteridge 2007). Although the degree to which this source of H_2O_2 contributes to oxidative stress under normal physiological circumstances is debatable, in certain disease conditions it may be significant (Beckman and Ames 1998).

Exogenous Sources of ROS

In addition to endogenous sources, exogenous action can also generate oxidants. Examples of this include exposure to UV or ionising radiation (Gerschman, Gilbert et al. 1954; Riley 1994; Mulero, Romeu et al. 2006), environmental toxins (Matsumura 2003), cigarette smoke (Church and Pryor 1985; Mayo, Kohlhepp et al. 2004) and chemotherapeutics (Muller, Niethammer et al. 1998).

1.1.2 Types of Oxidative Damage

Oxidative stress has been defined as a "disturbance in the pro-oxidant–antioxidant balance in favour of the former leading to potential damage" (Halliwell and Gutteridge 2007). This stress can result from a reduction in antioxidants or an increase in pro-oxidants (Halliwell and Gutteridge 2007). Persistent or extreme oxidative stress can eventually lead to cell death via apoptosis or necrosis (Fatokun, Stone et al. 2007; Ryter, Kim et al. 2007). Organisms have evolved complex antioxidant defence mechanisms to combat ROS and minimise oxidative damage and these will be discussed in more detail in Section 1.4.

Oxidative damage can be broadly grouped into three main types according to the biomolecule affected: damage to DNA, damage to proteins and damage to lipids.

DNA Damage

DNA damage includes specific damage to bases and degradation of deoxyribose residues (Marnett and Plastaras 2001). DNA assayed after exposure to pro-oxidants has been reported to contain an increased level of 8-hydroxydeoxyguanosine, a known radiation damage product (Richter, Park et al. 1988; Nygren, Ristoff et al. 2005). This increased level is believed to result from a reaction between the hydroxyl radical and purines (Cadet, Delatour et al. 1999). DNA exposed to oxidative stress also demonstrates an increased level of strand breakage and cross linkage (Schraufstatter, Hyslop et al. 1988; O'Neill and Fielden 1993; Hasty, Campisi et al. 2003; Kim 2004). Mitochondrial DNA is thought to be a critical cellular target for ROS. Damage to mitochondrial DNA is reported to be more extensive and persist longer than nuclear DNA damage (Jakes and van Houten 1997) and this can lead to a positive damage feedback loop with increased mitochondrial DNA damage giving rise to increased ROS which then cause further mitochondrial DNA damage. Often oxidative damage results in the cessation of DNA replication, enabling repair mechanisms to come into play. Base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR) have all been implicated as mechanisms dealing with the repair of oxidative damage to DNA (Langie, Knaapen et al. 2007; Russo, De Luca et al. 2007).

Protein Damage

Oxidative stress also contributes to a wide spectrum of protein damage including modifications of the polypeptide backbone, nucleophilic side chains and redox-sensitive side chains (Dean, Fu et al. 1997; Claiborne, Yeh et al. 1999; Marnett, Riggins et al. 2003). Being structurally flexible and possessing reactive amino acid residues makes proteins particularly vulnerable to this kind of damage (Stadtman and Levine 2003). In addition, oxidation reactions involving methionine residues are a common result of oxidative stress (Levine, Moskovitz et al. 2000).

Lipid Damage

Lipid peroxidation caused by the action of ROS on polyunsaturated fatty acids has been implicated in the pathology of certain age-related diseases (Smith, Mitchinson et al. 1992; Halliwell and Chirico 1993; Spiteller 2001) and a correlation has been reported between lipid peroxidation and DNA damage (Fraga and Tappel 1988). Membranes are particularly vulnerable to this kind of oxidative damage, with a high lipid content and a high proportion of unsaturated fatty acids (Yu, Masoro et al. 1982; Sohal and Weindruch 1996). This leads to a damaging positive feedback situation as membranes, particularly the mitochondrial membrane, are major sites of ROS production.

1.2. The Role of Reactive Oxygen Species in Normal Biology

While it is important that an organism's antioxidant defence mechanisms act in such a way as to minimise the imbalance between pro- and antioxidants, there is evidence that ROS do

themselves perform roles in certain physiological processes such as cellular proliferation and host defence. Therefore, it is rather a state of dynamic homeostasis that is optimal for an organism, enabling normal growth and metabolism, than a complete eradication of pro-oxidant molecules (Finkel and Holbrook 2000). The question remains as to why this homeostatic balance is of benefit to an organism. Several reasons have been proposed including the possibility that the energetic cost of repair or cellular destruction is less than the elimination of all ROS (Halliwell and Gutteridge 2007). It is also possible that complete protection is impossible and organisms have evolved a 'tolerance' for these molecules at certain levels. Whilst both of these are likely to be involved, it is clear that small amounts of ROS serve useful physiological functions.

ROS are known to play a significant role in cell signalling at many levels. Several protein tyrosine kinases and protein tyrosine phosphatases exhibit redox sensitivity and, therefore, ROS can influence the phosphorylation status of proteins, a key factor in many signalling cascades (Covarrubias, Hernandez-Garcia et al. 2008). ROS are also intimately linked to the action of a variety of transcription factors. They have long been known to play a vital role in post-translational modification, either by their part in pathways that determine phosphorylation status or via specific redox-regulation of transcription factors themselves (Keogh, Allen et al. 1998; Allen and Tresini 2000). The role of oxidant molecules in the regulation of transcription is reviewed in more detail in Section 1.5.4 in relation to Nrf-2, Keap1 and MAPK signalling cascades. In more recent years, their involvement in transcriptional activation has also been reported (Covarrubias, Hernandez-Garcia et al. 2008). H_2O_2 produced by receptor-ligand interactions between members of the

haematopoietin receptor superfamily and the EGF receptor acts as a chemical mediator facilitating signalling (Covarrubias, Hernandez-Garcia et al. 2008). In addition, redox-sensitive inflammatory signalling in microglia and the initiation of neurotoxic inflammation are dependent on the superoxide radical (Dimayuga, Wang et al. 2007).

Another signalling function fulfilled by ROS is that of the regulation of proliferation and the triggering of signalling pathways leading to cell death by apoptosis, autophagy or necrosis. H_2O_2 is known to stimulate a proliferative response in a variety of tissue types at low levels (Burdon, Gill et al. 1989; Burdon, Gill et al. 1990; Rao and Berk 1992; Ruiz-Ginés, López-Ongil et al. 2000). Increasing levels of ROS can induce cell death in a level-dependent manner with low, intermediate and high levels triggering cell death via apoptosis, autophagy and necrosis respectively (Kroemer, Petit et al. 1995; Schulz, Weller et al. 1996; Pelicano, Feng et al. 2003; Sagi, Wolfson et al. 2005; Scherz-Shouval, Shvets et al. 2007; Scherz-Shouval, Shvets et al. 2007).

These proposed roles are relevant from the perspective of experimental manipulation of individual components of the oxidative defence system as described in this thesis. Whilst this manipulation may provide a beneficial level of protection against the negative aspects of oxidative insult, it must be examined in the context of the whole organism in order to assess whether this benefit comes at a cost (i.e. the loss of certain positive roles that ROS play in the physiology of the whole organism).

1.3. Oxidative Stress and Disease

Aberrant expression of some antioxidant defence system genes leads to disease conditions with severe pathophysiology. Mutations in the gene that encodes CuZnSOD are responsible for about 20% of cases of familial amyotrophic lateral sclerosis (FALS), the inherited form of ALS (Halliwell and Gutteridge 2007). ALS is a late-onset neurological disease characterized by progressive loss of motor neurons (Keller, Mathieu et al. 2009). Disease pathology is associated with accumulation of aggregated forms of the mutant protein (Karch, Prudencio et al. 2009) and different SOD mutations are associated with different rates of disease progression (Halliwell and Gutteridge 2007) and act via different mechanisms (Muller, Liu et al. 2008). Reduced levels of GSH are associated with a variety of human diseases including Parkinson's disease, Wilson's disease, Kwashiorkor and idiopathic pulmonary fibrosis (Halliwell and Gutteridge 2007) although it remains to be determined whether this is a causative factor or an associated effect. However, defective expression of some components of the antioxidant defence system, such as catalase, appears to have little or no detectable clinical effects, implying that there is a degree of compensatory redundancy inherent in the system. An example of this is acatalasaemia, a relatively mild condition resulting from mutations in the catalase gene in humans (Halliwell and Gutteridge 2007).

In addition, elevated levels of ROS have been reported in conjunction with many disease conditions including autoimmune disorders such as rheumatoid arthritis, disorders of the eye such as macular degeneration, hepatitis and other diseases of the GI tract, kidney disorders, lung conditions such as cystic fibrosis and pulmonary fibrosis, neurodegenerative

disorders including Parkinson's, Huntington's, Alzheimer's and multiple sclerosis, diseases of the skin such as contact dermatitis and psoriasis, diseases of the vascular system and cancer (Phillips, Campbell et al. 1989; Abdollahi, Ranjbar et al. 2004; Dugan and Quick 2005; Reliene and Schiestl 2005). These elevated levels are not, however, conclusive proof that the ROS are in any way causative in these conditions. It is possible that these increased levels are a result of the condition itself and any suggestion of direct causation should be treated with caution. It remains a challenge for researchers in this area to isolate and elucidate the exact relationship between ROS and the conditions they are associated with.

1.4. Antioxidant Defence System

Organisms have a robust antioxidant defence system which counteracts the production and effects of ROS in a variety of ways. Halliwell and Gutteridge describe an antioxidant as "any substance that delays, prevents or removes oxidative damage to a target molecule" (Halliwell and Gutteridge 2007). An organism's defence system can be broadly divided into three main types of protection: pre-emptive defensive mechanisms minimise the formation of reactive species, the primary defence system deals mainly with the oxidant molecules themselves and a secondary defence system encompasses defences that give protection from and repair damage caused by ROS. The relative importance of individual antioxidant defences depends on which ROS are generated, how and where they are generated and what the target of the damage is.

1.4.1 Pre-Emptive Defence Mechanisms and Molecules

The formation of reactive species is minimised by mechanisms that decrease the formation of ROS directly, such as mitochondrial uncoupling or by molecules that minimise the availability of pro-oxidants including iron ions, copper ions and haem such as transferrins, albumin, haptoglobins, haemopexin, metallothionein and proteins that oxidise Fe^{2+} (Halliwell and Gutteridge 2007).

1.4.2 Primary Antioxidant Defences

The primary antioxidant defence system includes antioxidant enzymes such as SOD and catalase and specific ROS scavengers such as glutathione. These primary defences constitute a significant cellular response to oxidative stress, detoxifying ROS. Some of the most important defence enzymes and the reactions that they catalyse are listed in Table 1.2 below.

Table 1.2: Antioxidant enzymes and the reactions that they catalyse (Halliwell and Gutteridge 2007)

Enzyme	Reaction	Description
Superoxide dismutase (SOD)	$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$	Accelerates catalytic removal of $O_2^{\bullet -}$ (dismutation reaction)
Catalase	$2H_2O_2 \rightarrow 2H_2O + O_2$	Catalyses direct decomposition of H_2O_2 to ground state O_2 (dismutation reaction)
Peroxidases	$SH_2 + H_2O_2 \rightarrow S + 2H_2O$ (S = substrate)	Removes H_2O_2 by using it to oxidise another substrate
Glutathione peroxidase family	$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$	Removes H_2O_2 by coupling reduction to H_2O with oxidation of reduced glutathione (GSH) Specific for GSH as a hydrogen donor
Glutathione reductase	$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$	Conversion of oxidised to reduced glutathione

SOD and catalase constitute the primary enzymatic defence in *Drosophila* (Mackay and Bewley 1989; Radyuk, Klichko et al. 2001; Mockett, Bayne et al. 2003). Whilst catalase is encoded by a single gene in *Drosophila* (Mackay and Bewley 1989), SOD, the enzyme that catalyses the dismutation of $O_2^{\bullet -}$ to H_2O_2, exists in two forms, encoded by two genes. The cytosolic form, CuZnSOD, has copper and zinc ions at the active site. The mitochondrial form, MnSOD, has a manganese ion at the active site. (Phillips, Campbell et al. 1989; Orr and Sohal 1993; Halliwell and Gutteridge 2007). Despite imperfect removal of $O_2^{\bullet -}$ through SOD-mediated dismutation, these enzymes provide sufficient protection in *Drosophila* to enable survival (Seto, Hayashi et al. 1990; Phillips, Parkes et al. 2000). The role of SOD in lifespan determination in *Drosophila* is examined in more detail in Section 1.8.2.

In addition to primary enzymatic defences, organisms also have non-enzymatic ROS scavenging molecules which complement the anti-oxidant enzymes in neutralising oxidative stress. The role of the most important of these, glutathione, is reviewed in detail in Section 1.5.

1.4.3 Secondary Antioxidant Defences

Organisms also possess secondary defences that act to repair damage resulting from ROS-induced stress. These include systems that repair damage to DNA, such as MTH1 (mut T homologue 1) in mice which hydrolyses 8OHGTP leading to eventual secretion, or enzymes that repair oxidative protein damage such as MSR (methionine sulphoxide

reductase) which is involved in the repair of methionine residues (Halliwell and Gutteridge 2007). Where repair is not possible, damaged proteins are removed via protein degradation systems contained in organelles such as the lysosomes where hydrolytic enzymes degrade the damaged proteins taken in via endocytosis, or the proteasome, a system responsible for the removal of oxidatively damaged proteins (Halliwell and Gutteridge 2007).

1.5. Glutathione

1.5.1 Glutathione: A Master Antioxidant

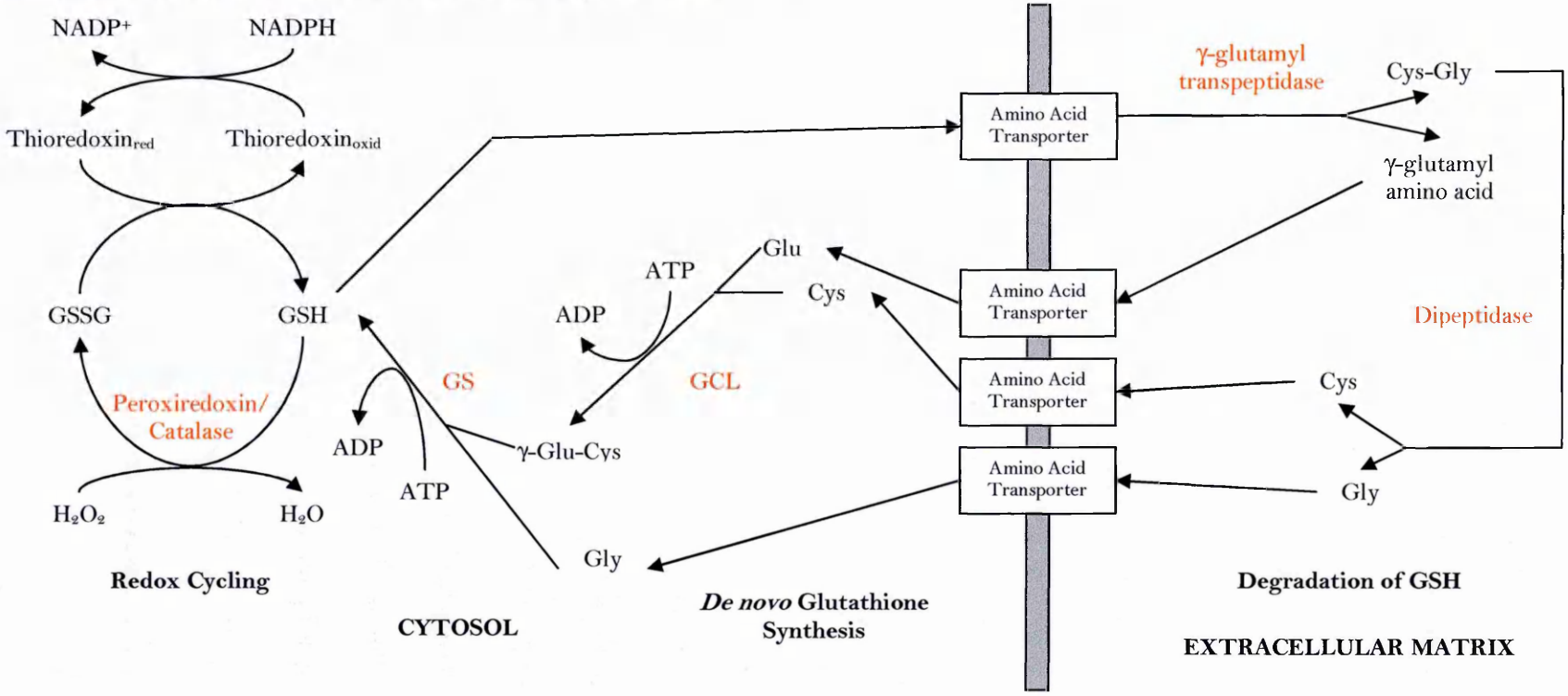
In addition to the antioxidant enzymes discussed above, organisms also possess non-enzymatic antioxidant molecules, which can remove ROS and hence offer protection from oxidative damage. The tripeptide glutathione (GSH) is the most important low molecular weight non-enzymatic antioxidant synthesised in cells (Meister 1992; Rebrin, Bayne et al. 2004; Forman, Zhang et al. 2009) and the most important non-protein thiol. It is recognised as a key component of the antioxidant defence system.

Although glutathione is widely distributed across all cell types, concentrations vary between individual cellular compartments creating distinct redox micro-environments within organelles (Rebrin and Sohal 2008). In mammals, it is present in the cytosol of cells where synthesis occurs at concentrations of 1-10mM (rats) (Forman, Zhang et al. 2009). Levels in the plasma are lower (in the micromolar range) as a result of its metabolism by other cells in rat liver (Sies and Graf 1985). In certain areas where high levels of oxidative stress result

from processes such as gas exchange, for example the fluid lining of the lung, glutathione concentrations are high, secreted by epithelial cells in humas (Cantin, North et al. 1987). However, it is found in highest concentrations in hepatocytes which export GSH to the plasma where it is used as a source of cysteine for GSH synthesis in other cells in humans and rats (Anderson and Meister 1980; Forman, Zhang et al. 2009).

Glutathione is required for survival in animals. Chemically induced GSH deficiency in rats and guinea pigs leads to multi-organ failure and death (Mårtensson and Meister 1991; Mårtensson, Stole et al. 1991). Specific cellular damage is seen in mitochondria and other structures (Meister 1994). These effects are not seen when rats are given a cellular GSH delivery agent simultaneously with the chemical depletor (Mårtensson, Steinherz et al. 1989). In addition, high glutathione levels in the blood have been reported to correlate with good mental and physical health in humans (Lang, Mills et al. 2002). The GSH-NADPH system is the main provider of reducing power in cells and constitutes a major antioxidant system for the elimination of ROS (Rebrin and Sohal 2008). As thiol-disulphide exchange reactions are rapid and readily reversible, this system is ideally suited to redox control of protein structural and catalytic function (Cho, Kim et al. 2003). In conjunction with the thioredoxin system, GSH plays a role in the regulation of the signal transduction activity of several kinases and phosphatases, thereby regulating redox control of cell growth, death and transactivation of redox sensitive transcription factors (Cho, Kim et al. 2003).

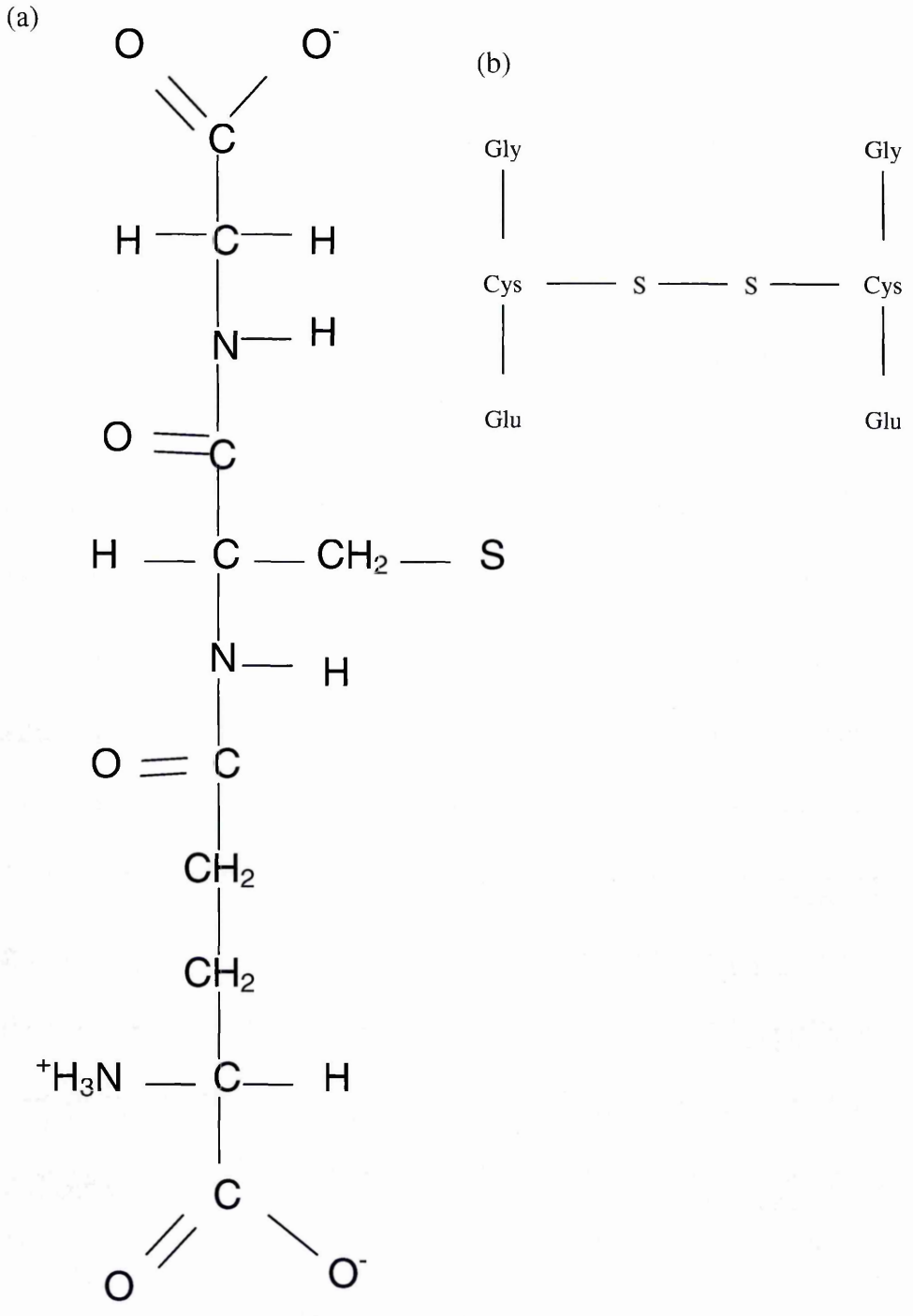
Figure 1.2 Glutathione synthesis and redox cycling in *Drosophila*. *De novo* glutathione synthesis occurs in the cytoplasm as a two-step ATP-dependent sequential reaction. The rate-limiting step, catalysed by the enzyme GCL, involves the ligation of L-glutamate and L-cysteine and is coupled to ATP hydrolysis, required to form the amide bond between the -carboxyl group of glutamate and the amino group of cysteine. The second step, catalysed by the enzyme GS involves the addition of glycine to -glutamylcysteine. GSH is then transported across the cell membrane to the extracellular matrix where it is eventually cleaved in a two-step reaction to form glutamyl amino acids, glycine and cysteine, which are in turn transported into the cytosol where they take part in *de novo* glutathione synthesis. In addition, cytosolic glutathione functions as an antioxidant, protecting the cell against ROS and H_2O_2. This is achieved via redox cycling between GSH and GSSG, catalysed in *Drosophila* by both peroxiredoxin and catalase. Oxidised glutathione is subsequently reduced via the thioredoxin system in *Drosophila*, replenishing the cellular pool of GSH. In mammals, the function of the peroxiredoxin system is carried out by glutathione reductase and that of peroxiredoxin by glutathione peroxidase. These enzymes are not present in *Drosophila*. (Meister 1992; Krzywanski, Dickinson et al. 2004; Rebrin, Bayne et al. 2004; Toroser, Yarian et al. 2006; Rebrin and Sohal 2008; Forman, Zhang et al. 2009)



De novo glutathione synthesis occurs in the cytoplasm as a two-step sequential reaction as shown in Figure 1.2. This is a necessary component of the cellular glutathione cycle as the cellular GSH pool maintained by the GSH renewal process involving thioredoxin or glutathione reductase does not compensate for the depletion of GSH via detoxification reactions and direct free radical scavenging. In mammals, despite the capacity for *de novo* synthesis in most cells, the liver remains a source of GSH for use by other organs (transported via the bloodstream) and the gastrointestinal system (transported via the bile) (Griffith and Meister 1979; Franklin, Backos et al. 2009). The sequential addition of cysteine to glutamate catalysed by GCL, followed by the addition of glycine catalysed by GS takes place in the cytosol (Figure 1.2). Glutathione is then transported to the extracellular matrix where it undergoes sequential cleavage giving rise to precursor amino acids which are in turn transported to the cytosol where they are available for *de novo* glutathione synthesis. Glutathione constantly cycles between a reduced (GSH) and oxidised (GSSG) state (Figure 1.3). As the GSH:GSSG redox couple is 3-4 orders of magnitude more abundant than other redox couples, it is the primary intracellular determinant of redox state (Bauer, Kanzok et al. 2002; Rebrin and Sohal 2008). In addition, it has a lower standard redox potential (E_0=240 mV) than other redox couples (Rebrin and Sohal 2008) which facilitates rapid cycling between GSSG and GSH in the reduction/oxidation reaction shown below:

$$2GSH \longrightarrow GSSG + 2e^- + 2H^+$$

Figure 1.3 The structure of (a) reduced (GSH) and (b) oxidised (GSSG) glutathione (Halliwell and Gutteridge, 2007)



In mammalian cells, glutathione reductase (GR) catalyses the reduction of GSSG to 2GSH. In *Drosophila*, GR is not present. Instead GSSG is reduced back to GSH by the thioredoxin system as illustrated in Figure 1.2 (Kanzok, 2001). Despite the potential for this ratio to shift towards toxicity, high glutathione reductase activity in mammals and the thioredoxin system in *Drosophila* combined with some secretion, maintain a GSSG:GSH ratio that is not toxic to cells (Wu, Fang et al. 2004; Toroser, Yarian et al. 2006; Forman, Zhang et al. 2009).

The redox potential of the GSH:GSSG couple is dependent on both the total amount of GSH and GSSG and their relative ratios (Rebrin and Sohal 2008). The GSH:GSSG ratio is commonly used as a measure of the level of oxidative stress in a cell with a decrease indicating an increase in oxidative stress (Frosali, Di Simplicio et al. 2004; Rebrin and Sohal 2008). The thiol group (-SH) is responsible for the potent reducing properties of GSH. In addition, GSH has high electron donating ability (Nygren, Ristoff et al. 2005) and is involved in the neutralisation of H_2O_2 to H_2O, a reaction which is catalysed by peroxiredoxin and catalase in *Drosophila* (Radyuk, Klichko et al. 2001) and by glutathione peroxidase in mammals. GSH is also capable of direct interaction with $OH^{\bullet}$ facilitating its reduction to H_2O. The combination of these direct interactions alongside GSH's ability to repair damage to macromolecules via the donation of a hydrogen atom, makes GSH one of the most powerful antioxidants in the cellular arsenal (Meister 1992; Nygren, Ristoff et al. 2005).

In addition to its antioxidant properties, GSH also plays an important role in the elimination of xenobiotic compounds via conjugation both non-enzymatically and via enzymatic reactions catalysed by glutathione S-transferase (GST) (Boyland and Chasseaud 1968; Meister 1992; Halliwell and Gutteridge 2007). GSH is involved in a variety of other cellular processes in addition to antioxidant defence including acting as a co-factor and a substrate for other antioxidant enzymes, prevention of protein cross-linkage, nucleic acid synthesis, leukotriene synthesis, cell cycle regulation, amino acid transport, Ca^{2+} homeostasis, cell cycle regulation and signal transduction activity (Meister and Tate 1976; Kosower and Kosower 1978; Parker, Fischman et al. 1980; Fischman, Udey et al. 1981; Shaw and Chou 1986; Meister 1988; Lu and Ge 1992; Kehrer and Lund 1994; Janáky, Ogita et al. 1999; Polekhina, Board et al. 1999; Halliwell and Gutteridge 2007; Kabil, Partridge et al. 2007; Forman, Zhang et al. 2009)

GCL

Three factors combine to regulate GSH homeostasis: the rate of GSH synthesis, the rate of GSH utilisation and the rate of GSH export (Griffith 1999). The capacity to synthesise GSH is controlled by the availability of its substrates, in particular cysteine, and the activity of the rate limiting enzyme in GSH synthesis, GCL (Griffith 1999). In eukaryotes, GCL is a heterodimer comprising a 73kDa catalytic subunit (GCLC) and a 31kDa modifier subunit (GCLM). GCLC has an active site responsible for ATP-dependent bond formation between the amino group of cysteine and the γ-carboxyl group of glutamate (Huang, Chang et al. 1993; Misra and Griffith 1998; Tu and Anders 1998; Franklin, Backos et al. 2009) and was originally cloned in *Drosophila* via functional complementation

in yeast (Saunders and McLellan 2000). GCLM possesses no independent catalytic activity but instead increases the catalytic efficiency of GCLC via direct interaction, lowering the K_m for glutamate and ATP and increasing the K_i for GSH feedback inhibition (Griffith 1999; Yang, Dieter et al. 2002). GSH inhibits GCL via competition with glutamate for the active site of GCLC (Franklin, Backos et al. 2009). Identified in 2002 by Fraser and colleagues, *Drosophila* GCLM complexes with GCLC via the formation of reversible disulphide bridges creating a catalytically more efficient holoenzyme (Fraser, Saunders et al. 2002). When complexed, it reduces GCLC sensitivity to GSH inhibition possibly as a result of conformational changes that prevent glutathione access to the active site (Huang, Chang et al. 1993; Fraser, Saunders et al. 2002).

Gclc and *Gclm* are separate genes on separate chromosomes giving rise to 2 distinct gene products in humans, mice and *Drosophila* (*Gclc* is located at 6p12 in humans, 9D-E in mice and X7D6-7D6 in *Drosophila*, whilst *Gclm* is located at 1p22-1 in humans, 3H1-3 in mice and 3R 94C1-94C1 in *Drosophila*) (Sierra-Rivera, Summar et al. 1995; Walsh, Li et al. 1996; Saunders and McLellan 2000). Holoenzyme formation occurs via the formation of intermolecular disulphide bridges (Farrington, Ebert et al. 1973; Tu and Anders 1998; Fraser, Saunders et al. 2002; Fraser, Kansagra et al. 2003). Cysteine residues have been found to play a role in regulating holoenzyme formation and activity in both human GCLC (Tu and Anders 1998) and *Drosophila* GCLM (Fraser, Kansagra et al. 2003). In *Drosophila*, 3 key cysteine residues on GCLM have been identified as important for inter-subunit disulphide bond formation (Cys^{213}, Cys^{214}, Cys^{267}) (Fraser, Kansagra et al. 2003). Two of these are conserved within human GCLM (Cys^{193}, Cys^{194}). Mutation of these

residues inhibited disulphide bond formation but not subunit association and GCLM mutant for these key cysteines enhanced GCLC activity at a reduced level (Fraser, Kansagra et al. 2003). This implies that the disulphide bonds induce a conformational change that makes the holoenzyme complex more catalytically efficient, possibly facilitating greater substrate uptake, rather than affecting the binding of the two sub-units. Reduction of inter-subunit disulphide bonds results in a conformational change that affects accessibility to the substrate binding site (Huang, Chang et al. 1993 ; Fraser, Saunders et al. 2002; Fraser, Kansagra et al. 2003). Sub-unit dissociation is not induced even at high GSH concentrations (Tu and Anders 1998; Yang, Dieter et al. 2002).

Glutathione is predominantly synthesised in the cytoplasm and levels vary between intracellular compartments such as the nucleus, mitochondria, endoplasmic reticulum and cytosol (Bellomo, Vairetti et al. 1992; Chen and Lash 1998; Dixon, Heath et al. 2008). There are two putative mechanisms for GSH distribution: *de novo* synthesis in the cytoplasm with subsequent transportation to different organelle types and in organelles with GCL and GS activity, synthesis *in situ* (Radyuk, Michalak et al. 2009). In culture, GSH levels are higher in the nucleus of proliferating cells compared to nuclear levels in confluent cells (Markovic, Borrás et al. 2007). *In silico* analysis predicts that *Drosophila* GCLC has a 74% probability of nuclear localisation, resulting from the presence of a nuclear localisation signal (NLS), whilst GCLM has a 52% probability of cytosolic location (Radyuk, Rebrin et al. 2009). This localisation pattern has been confirmed by *in vitro* and *in vivo* work (Radyuk, Rebrin et al. 2009). Radyuk and colleagues (2009) propose that GCLC plays a role as a nucleocytoplasmic shuttling protein, localising to the nucleus

during cell proliferation or in response to glutathione depletion (by cadmium treatment). The resultant increase in GSH level and pro-reducing shift in glutathione redox state creates a more reducing environment, preventing DNA damage and consequent cell cycle arrest (Markovic, Borrás et al. 2007; Radyuk, Rebrin et al. 2009). The localisation of GCLM to the cytoplasm may keep nuclear GSH levels lower than cytoplasmic levels.

Mutations in the GCLC subunit are associated with disease (Ristoff, Augustson et al. 2000; Mañú-Pereira, Gelbart et al. 2007). *Gclc*-null mice show embryonic lethality although heterozygotes showing 50% reduction in protein levels have a normal phenotype (Dalton, Dieter et al. 2000; Shi, Osei-Frimpong et al. 2000). Post-embryonic GCLC deletion leads to death resulting from extensive liver failure (Chen, Yang et al. 2007). In contrast, GCLM deletion in mice has little or no effect on survival or development despite reduced GSH levels (Yang, Dieter et al. 2002; McConnachie, Mohar et al. 2007). This absence of a noticeable effect on survival and viability is replicated in *Drosophila* (Fraser, Kansagra et al. 2003), implying that glutathione levels generated by the catalytic subunit alone are sufficient for viability.

The cellular response to oxidative stress can affect GCL in one of two ways: at a transcriptional level, it results in an increase in levels of GCL and at a post-transcriptional level there can be increased activity of the individual subunits (Griffith 1999; Franklin, Backos et al. 2009). Whilst a co-ordinate induction of both subunits occurs in response to oxidative stress, transcriptional and post-transcriptional mechanisms mediate differential

rates and levels (Yao, Godwin et al. 1995; Cai, Huang et al. 1997; Galloway, Blake et al. 1997). In the majority of cell types, it is actually GCLM that is limiting for holoenzyme formation (Krzywanski, Dickinson et al. 2004; Chen, Shertzer et al. 2005; Lee, Kang et al. 2006). This means that increased expression of GCLM alone is likely to be an effective mechanism for enhancing cellular GCL activity (Lee, Kang et al. 2006; Franklin, Backos et al. 2009). Increased activity can occur through post-translational modification of pre-existing GCLC or GCLM protein (Franklin, Backos et al. 2009).

1.5.2 Ageing and Glutathione Redox State

Ageing is associated with an increase in oxidative damage products and lipid peroxidation end-products such as malondialdehyde and hydroxynonenals which form adducts with DNA and proteins (Esterbauer, Schaur et al. 1991) in addition to increased formation of protein mixed disulphides which reduce the catalytic efficiency of enzymes and hence the ability to mount adaptive response under stress (Droge 2003; Rebrin, Bayne et al. 2004; Rebrin and Sohal 2008). Glutathione redox state is, therefore, vital as a limiting factor for this kind of macromolecular damage during the ageing process. During ageing, glutathione redox state can be affected by an increase in GSSG concentration and a decrease in the GSH pool, coupled with age-related increases in mitochondrial superoxide and H_2O_2 production this leads to enhanced age-related oxidative stress (Sohal, Mockett et al. 2002). In addition, steady-state GSH concentration declines in some tissues alongside a loss of catalytic activity of GCL and a lower affinity for substrates (Squier 2001; Toroser, Yarian et al. 2006; Toroser, Orr et al. 2007).

In mice, ageing is associated with a pro-oxidising redox shift in all organs, with increased levels of GSSG, decreased GSH content, decreased GSH:GSSG ratio and increased protein-SSG levels (Rebrin, Kamzalov et al. 2003). Different tissues have unique redox states and mitochondrial GSH levels, GSH:GSSG ratios and glutathione redox potentials are reduced relative to the corresponding tissue homogenate and hence more oxidising, implying greater sensitivity to age-related glutathione depletion (Rebrin, Kamzalov et al. 2003; Rebrin and Sohal 2008). In mouse brain, these age related shifts are specific to the forebrain and cerebellum and equivalent reductions are not seen in the brainstem (Rebrin, Forster et al. 2007). Rat brain also shows a pro-oxidising redox shift with lower GSH levels and higher GSSG levels leading to a reduced GSH:GSSG ratio. This is accompanied by reduced GCL activity and increased lipid peroxidation, alongside increased enzyme activity related to glutathione usage (GPx, -glutamyltranspeptidase and GST) (Zhu, Carvey et al. 2006). The specific age-related depletion of GSH and pro-oxidising shift in the GSH:GSSG ratio in neural tissue identifies it as a potential key, lifespan limiting tissue type.

Drosophila also show a pro-oxidising shift in GSH redox state alongside increased protein mixed disulphide. However, whilst the GSH:GSSG ratio decreases with age, GSH concentration remains unaffected and the shift comes from an increase in GSSG content (Rebrin, Bayne et al. 2004). Interestingly, increased ambient temperature and the subsequent reduction of lifespan in *Drosophila* is also accompanied by a pro-oxidising shift in redox state implying that increased temperature can be an effective model for accelerated ageing in *Drosophila* (Rebrin, Bayne et al. 2004). This pro-oxidising shift is accompanied

by a reduction in activity and gene expression of GCL in an organ-specific manner (Liu and Choi 2000).

1.5.3 Transcriptional Regulation of GCL – The Role of Antioxidant Response Elements (ARE)

On exposure to oxidants, for example H_2O_2, GSH levels show a characteristic depletion followed by a bounce-back increase after approximately 24 hours (Rahman, Biswas et al. 2005). This implies that there is a delayed signalling response to oxidant exposure in the GSH biosynthesis pathway. An example of transcriptional regulation of antioxidant gene expression occurs during the regulation via oxidant molecules of anti-oxidant responsive genes via *cis*-acting enhancer sequences known as EpREs or AREs (Nguyen and Pickett 1992). EpREs were first identified in rats from the promoter region of the rat GST Ya (Rushmore, King et al. 1990; Rushmore and Pickett 1990) and regulate a number of genes encoding stress responsive or cytoprotective enzymes and proteins such as SOD, GCL, GST GPx and catalase through mediation of expression due to the presence of an EpRE consensus sequence in the 5' flanking region of these genes (Yang, Dieter et al. 2002; Kim 2004; Rahman, Biswas et al. 2005). This is achieved through Nrf2-dependent activation resulting from either a Keap1 mediated mechanism (Figure 1.4) or serial phosphorylation cascades in the upstream MAPK signalling pathway (Figure 1.5) (Cobb and Goldsmith 1995; Kong, Owuor et al. 2001; Rahman, Biswas et al. 2005; Surh, Kundu et al. 2008).

Both human *Gclc* and *Gclm* genes contain EpRE enhancer sites in their promoter regions (Rahman, Biswas et al. 2005). In addition, *Gclc* also contains AP-1 and NF B binding sites, both redox-sensitive families of transcription factors (Rahman, Biswas et al. 2005). Nrf-2 is a member of the NF-E2 family of basic leucine zipper transcription factors known as cap 'n' collar proteins (Iles and Liu 2005). Under reducing conditions, Nrf-2 complexes in the cytoplasm with Keap1 (Itoh, Wakabayashi et al. 1999), a cysteine-rich homodimeric, multi-domain zinc metalloprotein anchored to the actin cytoskeleton (Surh and Na 2008). Under normal physiological conditions, Keap1 associates with Cul3 and Rbx1, targeting Nrf-2 for ubiquitin-dependent degradation (Figure 1.4) (Zhang, Lo et al. 2005). Under conditions of oxidative stress, Nrf-2 is stabilised as the cysteine residues of Keap1 are modified causing a conformational change which prevents Cul3 ubiquitination (Lee and Surh 2005). Nrf-2 then translocates to the nucleus and transactivates EpRE-regulated genes such as *Gclc* (Figure 1.4). An alternate mechanism underlying nuclear translocation of Nrf-2 involves its dissociation via serine/threonine phosphorylation (Figure 1.4) (Lo, Li et al. 2006; Yuan, Xu et al. 2006). Once localised to the nucleus, Nrf-2 combines with other transcription factors, particularly the small Maf protein, and binds to the 5'-upstream *cis*-acting regulatory sequence EpRE (Itoh, Wakabayashi et al. 1999) hence driving gene expression downstream.

Gclc and *Gclm* are also activated in an Nrf-2-dependent manner via serial phosphorylation cascades in the MAPK signalling pathway stimulated by oxidative stress (Figure 1.5) (Cobb and Goldsmith 1995). This mechanism of activation has the advantage of being fast and reversible, enabling relatively rapid organismal response to stress.

Figure 1.4 Diagram showing proposed mechanisms of stress-induced regulation of GCLC, the catalytic sub-unit of GCL in mammals (Rahman, Biswas et al. 2005; Surh, Kundu et al. 2008).

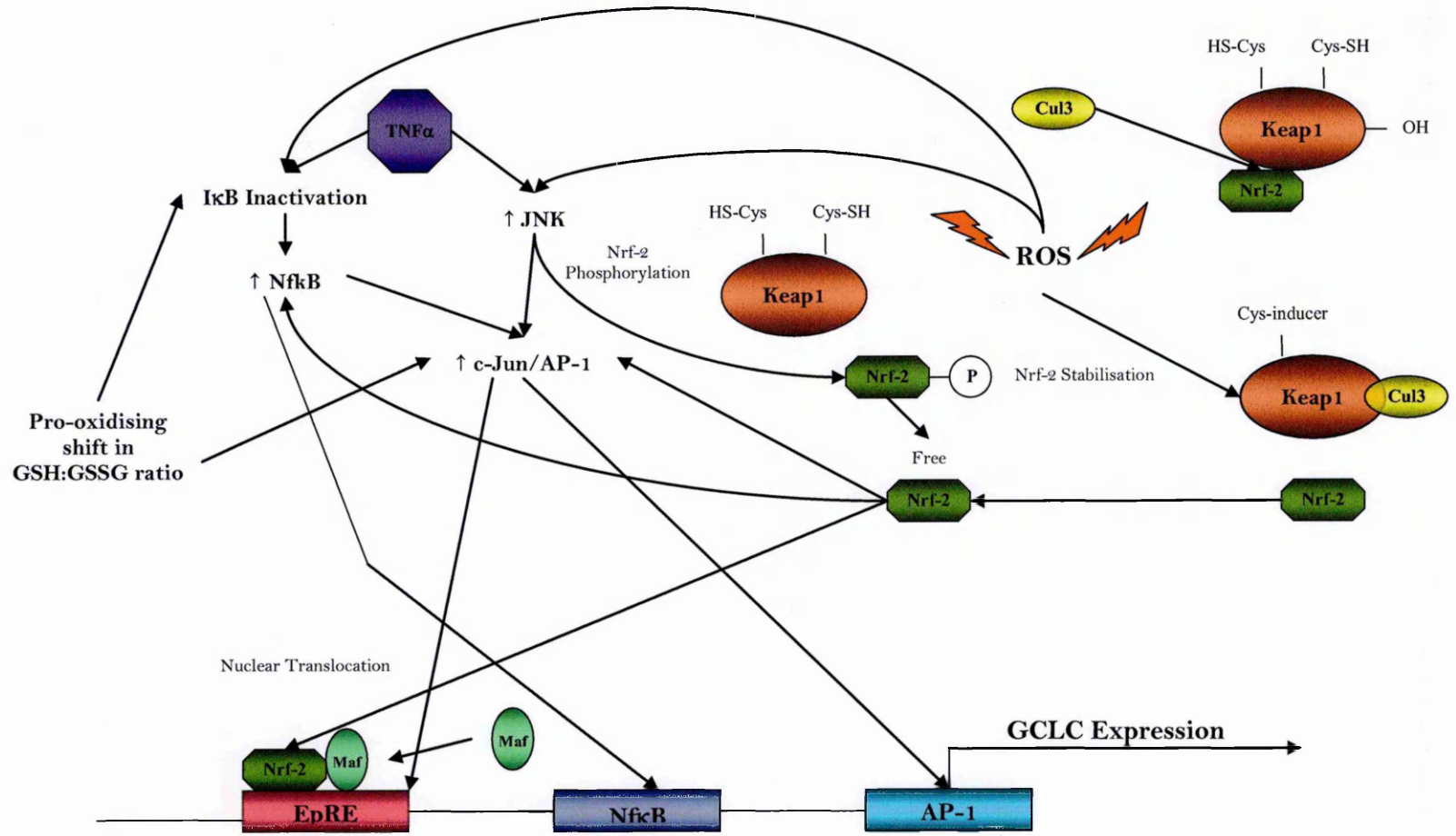
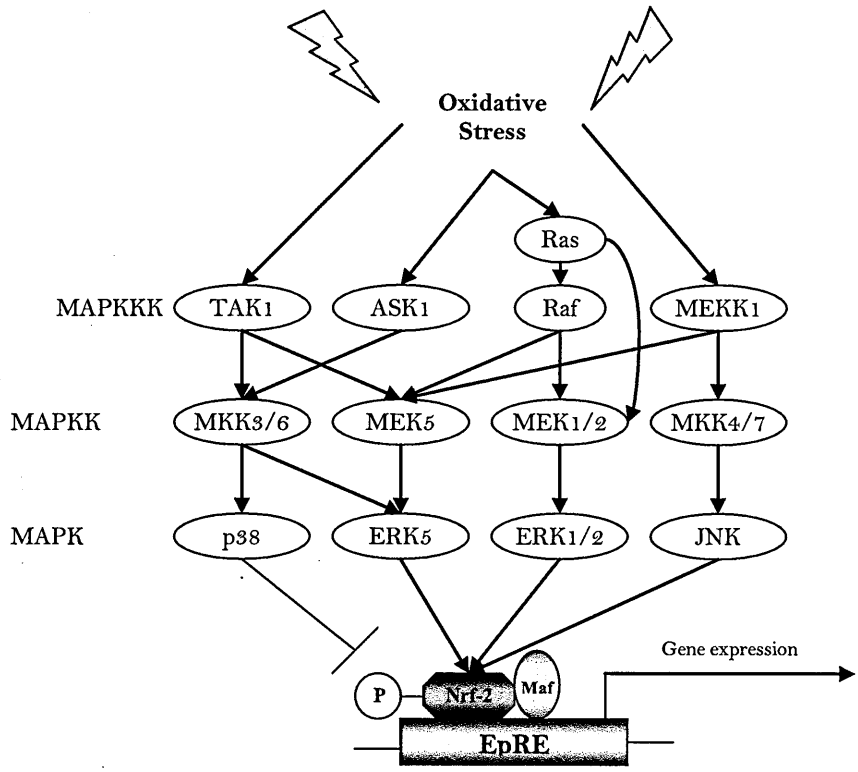


Figure 1.5 Proposed model of stress response leading to activation of the MAPK signalling pathway involving serial phosphorylation cascades in mammals (Cobb and Goldsmith 1995; Owuor and Kong 2002)



1.6. Theories of Ageing

Ageing is a complex phenomenon and this is reflected in the existence of a diverse range of theories as to the direct and indirect causes of the ageing phenotype.

1.6.1 What Is Ageing?

When discussing the possible connection between oxidative stress and ageing, it is first necessary to examine what is meant by the term 'ageing' as it is not a concept that is easy to define. It has been suggested that ageing is a progressive decline in the efficiency of physiological processes after the reproductive phase of life and that when formulating

possible ageing theories, they must take into account that there appears to be a 'fixed' maximum lifespan for a species, and the median lifespan can be modulated by external environmental conditions (Halliwell and Gutteridge 2007). There is also the suggestion that age-related diseases should be separated and discounted from studies of ageing itself and this concept does have validity. However, it must be borne in mind that 'ageing' is not a simple disease or condition but a multifactorial process intimately linked with physical decline, which includes a number of diseases that become more common with age. The dissociation of age-related disease and lifespan is difficult to achieve with this intimate linkage. It has been proposed that study of progeroid disorders such as Werner's Syndrome or Hutchinson-Gilford Syndrome serve as partial models for the ageing phenotype (Martin and Oshima 2000; Kudlow, Kennedy et al. 2007). This is a debatable point. These progeroid disorders are segmental and therefore do not show all symptoms associated with ageing. In addition, they are often accompanied by symptoms that are not seen during the normal course of ageing. Nonetheless, even with these reservations, these conditions could still provide information on some aspects of ageing and before discounting them as a source of information, it would be wise to remember the fact that as an organism ages, it develops a wide range of symptoms. The grouping of these under the general term 'ageing' is an artificial construct. Therefore, conditions such as Werner's Syndrome could provide an insight into a particular subset of these symptoms (Goto 1997). It is also important to bear in mind the fact that 'how' an organism ages is not merely a genetic issue, it also comprises epigenetic and environmental factors. Any combination of these factors can have a bearing on lifespan and survival at various life stages. This point is vital when considering

experimental investigation into causes of ageing, where it is crucial to disentangle environmental and genetic effects in order to accurately interpret data.

There are many diverse theories of ageing, indicative of the multifactorial nature of the condition. It is not possible to examine all of these in detail in this thesis. The review below focuses on the Free Radical Theory of Ageing as the work presented here is relevant to this theory in particular. There is also a brief review of the link between telomeres and ageing, in order to exemplify the complexity of the interaction between different theories with reference to the proposed link between *in vitro* telomere shortening and the application of different oxidative stressors.

1.6.2 Free Radical Theory of Ageing

One theory explaining the ageing phenomenon was proposed by Harman in 1956 (Harman 1956). ROS can cause damage to macromolecules and the repair of this damage is often incomplete. The basis of the Free Radical Theory of Ageing is that this incomplete repair process leads to an accumulation of damage in an organism. This accumulated damage is implicated in age-related deterioration seen in ageing organisms. Mitochondria subsequently became a focus of research into this theory as both free radical generators and targets of damage (Miquel, Economos et al. 1980; Fleming, Miquel et al. 1982; Miquel 1991).

There is certainly a large body of data supporting this theory as an explanation of the ageing process. The fact that the body's protection from oxidants is incomplete and that damage occurs is well-documented and it has been suggested that this damage could be exacerbated by increased ROS production as tissues age, leading to a situation where positive feedback causes an increasing cycle of self-perpetuating oxidative damage (Halliwell and Gutteridge 2007). As mitochondria are known to be an endogenous source of ROS (see Section 1.1.1), it would be predicted that any damage accumulation with age would be evident in these organelles. Papers by Lee (1997) and Yakes (1997) both show that mitochondrial DNA rapidly accumulates mutations with age (Lee and Wei 1997; Yakes and van Houten 1997). In addition, there is an inverse correlation between basal metabolic rates and lifespan. Perez-Campo *et al* (1994) reported that rats and pigeons have different lifespans but similar metabolic rates, which at first appears contradictory. However, mitochondria from pigeon tissue were shown to generate ROS *in vitro* at a slower rate than in rat which is consistent with the link between mitochondrial ROS production and lifespan (Barja, Cadenas et al. 1994). A study has shown considerable overlap between gene expression patterns in ageing and in hyperoxia-stressed flies (Landis, Abdueva et al. 2004). Another study into genome-wide transcript level changes and age in *Drosophila* has shown that when flies were treated with paraquat (a free radical generator) and their genome-wide transcript levels measured and compared to those of ageing flies, many of the same genes change in both cases (Zou, Meadows et al. 2000). This supports the predictions that the Free Radical Theory makes. However, there are genes which change when flies age that do not change with paraquat treatment. This implies that what happens with age is more complicated (Zou, Meadows et al. 2000). Even so, in cases where *Drosophila* have been

directly selected for paraquat resistance, a four-fold LT_{50} increase has been demonstrated (Vettraino, Buck et al. 2001).

Despite evidence in favour of the link between ROS and ageing, it is not a simple relationship. Work on mutants of the *methuselah* (*mth*) gene, a mutation which increases lifespan in *Drosophila* via an increase in oxidative stress resistance (Lin, Seroude et al. 1998), has shown that age-related functional decline in olfactory and locomotor systems is independent of the increased lifespan and stress resistance the *mth* flies demonstrate, with these flies showing no improvement in either lifespan or stress resistance when compared to control lines (Cook-Wiens and Grotewiel 2002). Different oxidative stressors also appear to have differing relationships to lifespan extension. In experiments with long- and short-lived lines of flies (selected for late or early reproduction), the long-lived flies have a shorter survival time on 100% O_2 than the short-lived lines, whilst this trend in survival time is reversed on exposure to paraquat (Mockett, Orr et al. 2001). The different modes of action of individual ROS and their differing effects on lifespan indicate further complexities in the relationship between ROS and ageing. Certain *Drosophila* lines that have demonstrated resistance to paraquat show no increase in lifespan at all, implying that stress resistance may be necessary but not sufficient for longevity (Harshman, Moore et al. 1999; Mockett, Bayne et al. 2003). In some cases, long-lived fly lines also show no increase in resistance to hyperoxia, supporting the difference in interaction between different oxidative stressors and an organisms antioxidant defence system (Mockett, Bayne et al. 2003).

In order to further elucidate the relationship between oxidative stress and ageing, certain elements of the antioxidant defence system have been manipulated and examined in a diverse range of model organisms including the nematode worm *Caernorhabditis elegans* (Ayyadevara, Dandapat et al. 2005; van Raamsdonk and Hekimi 2009), *Drosophila melanongaster* (reviewed below) and the mouse (Daoying, Caoa et al. 2006; Hu, Serrano et al. 2006). There is a large body of work concerning the effect of manipulation of levels of both cytosolic (CuZnSOD) and mitochondrial (MnSOD) superoxide dismutase enzymes. In both *D. melanogaster* and mouse genetic manipulations leading to a deficiency in both types of SOD leads to a reduction in lifespan and oxidative stress resistance (Harshman, Moore et al. 1999; Osterwalder, Yoon et al. 2001). However, this in itself does not prove that SOD is necessarily the most important factor in lifespan and stress resistance – a disruption of any biological system can have a negative effect on lifespan as a result of the negative effects of any extreme deviations from the biologically 'normal' state of an organism. In order to more clearly investigate whether SOD is involved in the ageing phenotype, it is necessary to see whether the over-expression of this enzyme has a positive effect on lifespan or stress resistance. Researchers addressing this question have adopted two approaches: genetic manipulation of organisms to engineer endogenous over-expression of SOD and the administration of exogenous SOD mimetics. In both cases the results have been contradictory.

The expression of the *Drosophila* CuZnSOD gene in *Escherichia coli* has been shown to confer resistance against paraquat-induced oxidative stress (Goulielmos, Arhontaki et al. 2003) and overexpression of both MnSOD and CuZnSOD ubiquitously in *D.*

melanogaster has been shown to increase lifespan (Sun and Tower 1999; Sun, Folk et al. 2002) and lifespan extension was also demonstrated when human *SOD1* was expressed in *D. melanogaster* motor neurons (Parkes, Elia et al. 1998). However, these results are not definitive and the absence of a full set of control genotypes in the lifespan assay casts some doubt on the validity of the extension shown. In addition, as only 2 responder elements were used in this study, there is a high likelihood that position effect of the transgene insertions could affect the results. The effect of genetic background on lifespan is also of crucial importance. Studies have shown that lifespan extension is often only seen in *D. melanogaster* lines which are originally short-lived (Orr, Mockett et al. 2003; Spencer, Howell et al. 2003) and is not replicated in long-lived strains. This is of vital importance when assessing the relevance of these results in the context of the role of certain genes and enzymes in the physiology of ageing. If the extension is only seen in a situation where the organism is in some way compromised and not in a background that is robust and long-lived and the source of the original lifespan differences cannot be discerned, it is impossible to state with any certainty whether or not over-expression of SOD is effecting lifespan via another, unconnected effect which is only relevant in compromised lines. This topic will be dealt with in more detail in Section 1.8.

The use of SOD/catalase mimetics has produced equally opposing results. In mice, SOD mimetics are reported to extend lifespan and reduce age-associated oxidative stress and mitochondrial radical production (Quick, Ali et al. 2008). In *C. elegans*, mean lifespan extension of 44% has been reported when these worms are treated with two SOD/catalase mimetics (EUK-8 and EUK-134 – synthetic, catalytic scavengers of ROS), and worms with

a premature ageing phenotype show a reversal of this phenotype with the same mimetics (Melov, Ravenscroft et al. 2000). SOD2 nullizygous mice treated with these mimetics also show a significant increase in mean lifespan (Melov, Doctrow et al. 2001). However, a separate study has failed to replicate the lifespan extension in *C. elegans* (Keaney and Gems 2003) and instead this study actually saw a dose-dependent reduction in both fertility and lifespan. These contrasting results highlight the importance of culture conditions in these experiments – it is possible that some subtle differences in culture conditions could completely remove any advantage that the mimetics provide the flies, or in fact that the culture conditions themselves could be responsible for the differences rather than the mimetics. Extension could be seen only in culture conditions or laboratory stock lines which are for some reason compromised or stressed as a result of genetic differences between laboratory populations accumulated over time under slightly different culture conditions. A study using the same mimetics in *Musca domestica* has also failed to show extension of mean lifespan and this implies that there is a certain level of species specificity in the effects of these mimetics (Bayne and Sohal 2002).

Summary of Current View of Free Radical Theory

Therefore, despite much research into these components of the antioxidant defence system, there are still serious questions that remain as to the role they play in ageing and lifespan. It is important to analyse these results in relation to other components in the system as no one portion of the defence systems exists in isolation. Manipulation of one component of the system can affect other biosynthetic pathways and interactions between enzymes and non-enzymatic components. The aim of this thesis is to look at the effects of manipulation

of certain components of the antioxidant defence system in *Drosophila melanogaster*, taking advantage of the capacity for *in vivo* studies presented by this model organism.

1.6.3 Telomeres and Ageing

In addition to the Free Radical Theory of Ageing, other explanations have been proposed for the ageing phenomenon, one of which implicates telomeres in the ageing process. It has been suggested that telomeric shortening could play a role in the ageing process (Goyns and Lavery 2000). Mammalian telomeres are composed of tandem repeat sequences of (TTAGGG)n (Meyne, Ratcliffe et al. 1989). The complete replication of the ends of eukaryotic chromosomes is achieved through the action of the enzyme telomerase, which acts as a template for the addition of telomeric DNA to the 3'-terminus of the ends of these chromosomes (Greider and Blackburn 1989). In the absence of telomerase, telomere shortening occurs and appears to be linked to the number of population doublings a cell can undergo before replicative senescence occurs (Shay and Wright 1991; Levy, Allsopp et al. 1992). Correlations have been observed between organismal ageing and *in vitro* population doublings in cell cultures from the same organisms (Rhome 1981) suggesting that telomeric shortening could be involved in the ageing process. However, it is difficult to disentangle cause and effect in these studies and so the suggestion that this shortening is, in fact, a consequence of the ageing process rather than a causal factor cannot be ruled out. As predicted by this theorised link, transfection of telomerase into human cell cultures did halt telomere loss (Bodnar, Oullette et al. 1998). However, *in vivo* the situation is not as clear-cut. Quiescent or post-mitotic tissue, which seldom proliferates, still ages despite an absence of telomere shortening (Goyns and Lavery 2000). Certain telomerase-negative cells (epithelial cells, fibroblasts) are not affected by telomere shortening as they do not undergo sufficient cell divisions in their lifetime and Goyns and Lavery suggest that this is evidence

that *in vitro* replicative senescence lacks relevance to the situation *in vivo* (Goyns and Lavery 2000). Telomerase deficient mice have been described as showing an accelerated ageing phenotype (Kipling and Faragher 1999) but, despite cells in early generation animals having shortened telomeres (Blasco, Lee et al. 1997), the accelerated ageing phenotype is not evident until the third generation of these animals. In addition, there is no apparent correlation between telomerase activity and *in vivo* ageing (Goyns and Lavery 2000). Therefore, whilst telomere shortening and ageing do appear to have some link, it is far from clear whether this is a direct causal link. It is yet to be fully proven that it is the shortening of telomeres themselves that give rise to the ageing phenotype and that this itself is not an observed effect of some other mechanism that leads to ageing in organisms.

The situation is further complicated by a proposed link between *in vitro* telomere shortening and the application of different oxidative stressors. This effect is reversed by treatment with certain antioxidants (von Zglinicki 2002). von Zglinicki proposes a purported causal role of oxidative stress in certain diseases could be explained by the link between telomere shortening and oxidative insult. This is not necessarily a satisfactory explanation of the situation in disease states where increased levels of oxidative damage are seen as it has not yet been clearly proven that the increase in oxidative stress and damage is not itself purely a result of the disease condition rather than a causal factor. Nonetheless, links like these show that when approaching the problem of oxidative stress and its effects on whole organisms, it is unwise to treat the oxidative response system as one that exists in isolation – there may be many interlinking systemic effects of manipulation of any one

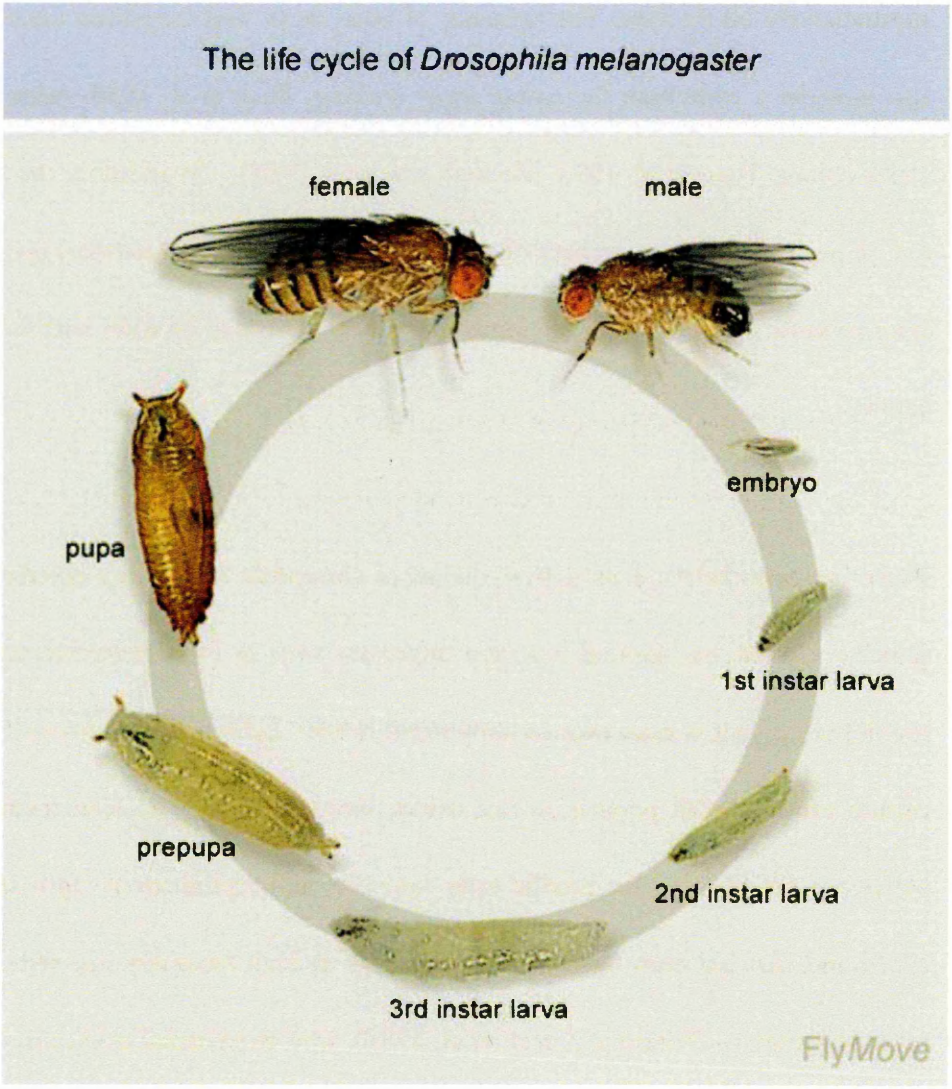
element of this system and it is necessary to keep the broad organismal picture in mind at all times.

1.7. *Drosophila melanogaster*: A Model Organism

1.7.1 Drosophila: An Effective Model System for the Study of Ageing and Stress Resistance?

Drosophila melanogaster is one of a variety of organisms that have been used as model systems to study ageing and stress resistance and has several advantages over *C. elegans* (Herndon, Schmeissner et al. 2002), *S. cerevisiae* (Guarente and Kenyon 2000) and rodents (Bartke, Wright et al. 2001). The *Drosophila* genome of approximately 180mb, encoding about 13,600 genes, was sequenced in 2000 (Adams, Celniker et al. 2000) The annotated version of this sequence provides a rich resource for researchers (Bernards and Hariharan 2001; Adams and Sekelsky 2002; Misra, Crosby et al. 2002; Drysdale and Crosby 2005), complemented by the wide range of genetic mutants available (Helfand and Rogina 2003). It is a relevant and vital resource for research into human disease conditions, with more than 70% of genes implicated in human disease having orthologues in *Drosophila* (Helfand and Rogina 2003). In addition, *Drosophila melanogaster* has a short generation time and this enables researchers to generate large populations of sibling flies for lifespan analysis. This provides a benefit over model organisms such as the rat or mouse as population numbers are sufficient to carry out large scale statistical analysis of survival. The relatively short lifespan of *Drosophila* (long-lived flies live approximately 90-100 days) means that replicate lifespan experiments can be carried out in over a sensible timescale.

Figure 1.6 Life cycle of *Drosophila melanogaster* (Weigmann, Klapper et al, 2003)



Drosophila has a well-defined life-cycle (Figure 1.6), passing through well-characterised embryonic stages and 3 larval instars before pupation and metamorphosis into a sexually mature adult over approximately 8-10 days and this facilitates developmental and temporal analysis. In addition, the fact that cells in the majority of *Drosophila* tissue types are post-mitotic (exceptions being the gonads and gastrointestinal tract) means that the effect of successive cell divisions on senescent changes is minimal (Helfand and Rogina 2003; Rebrin, Bayne et al. 2004). Relative to rodents models, *Drosophila* has a short lifespan

(Helfand and Rogina 2003), with average laboratory raised wild-type lifespan of approximately 60-80 days. The existence of both short- and long-lived laboratory strains also provides a wide basis for ageing study (Arking, Buck et al. 1988; Arking and Wells 1990; Arking, Force et al. 1996; Mockett, Orr et al. 2001). In addition, the fact that flies can be easily exposed to a variety of chemical and environmental stressors means that they are a versatile organism for these kinds of studies. This will be dealt with in more detail below.

There are, nevertheless, drawbacks to the use of *Drosophila*, as with any experimental model system. Genetic background is a very important issue in lifespan studies and can prove problematic. This is dealt with in more detail below. Laboratory conditions (regular early culture passage, small population size stocks, limited and defined food media) lead to a preference for selection for prolific early fecundity and reproduction (Sgro and Partridge 1999) and this has been cited as a disadvantage as high fecundity has been linked with reduced longevity (Partridge, Green et al. 1987), with the possibility that any increases in longevity shown by strains is merely a return to the normal levels seen in wild-type lines (Spencer, Howell et al. 2003). In addition, deleterious mutations that only affect older flies will accumulate under no selection pressure as parental flies are discarded after early laying in laboratory stock cultures (Spencer and Promislow 2002).

Despite the above, *Drosophila* remains a powerful tool for examining lifespan and stress resistance. In order to fully appreciate this, it is necessary to examine in more detail some of the genetic techniques that have been used in such studies.

1.7.2 P-Elements and Transgenic Techniques

P-elements are a class of transposable elements found in *Drosophila melanogaster* capable of moving within the *Drosophila* genome. Engineered P-elements are a genetic tool that takes advantage of the structure and characteristics of one class of transposable elements found in *Drosophila* (Georgiev 1984). These elements were originally identified as possible causes of sterility, male recombination and high mutability detected in certain wild-type strains of *D. melanogaster* (Hiraizumi 1971; Waddle and Oster 1974; Kidwell, Kidwell et al. 1977; Yannopoulos and Pelecanos 1977). As these elements were mapped to multiple locations it was suggested that they possessed mobility (Engels 1979; Engels and Preston 1980) and this characteristic has now been exploited to the advantage of fly geneticists, enabling them to use P-elements as gene transfer vectors and to induce mutations (Rubin, Kidwell et al. 1982; Rubin and Spradling 1982; Spradling and Rubin 1982).

P-elements were originally cloned in the 1980s (Bingham, Kidwell et al. 1982; Rubin, Kidwell et al. 1982). The molecular mechanism of P-transposition relies on the duplication of 8bp of chromosomal DNA on insertion. This production of a small repeat at the insertion site is characteristic of transposable elements as a whole (Calos and Miller

1980; O'Hare and Rubin 1983). P-elements are only transpositionally active in the presence of transposase, which can be supplied by other P-elements (Spradling and Rubin 1982). For this reason, P-element work is carried out in M-cytotype strains which lack their own functional P-elements (Spencer, Howell et al. 2003) – most laboratory strains are M-cytotype, having been collected prior to the rapid spread of P-elements in the wild *Drosophila melanogaster* population (Bingham, Kidwell et al. 1982; Kidwell 1982; Kidwell 1983). Engineered P-elements used in laboratories lack their own source of transposase and so are 'controllable' (Ryder and Russell 2003; Castro and Carareto 2004). Certain characteristics of P-elements should be noted. They show a wide range of affinity to individual target loci, with certain 'hot-spots', such as the *sn* locus, having a particularly high insertion percentage (Engels 1979; Rubin, Kidwell et al. 1982). Transposition is tissue-specific, limited to the germline (Thompson, Woodruff et al. 1978; Engels 1979; Bingham, Kidwell et al. 1982). In addition, their action is temperature-dependent, with a maximum frequency of mobilisation at 25°C (Bregliano, Picard et al. 1980). P-elements can be used to induce insertional mutations through direct insertion into genes (Simmons and Lim 1980; Rubin, Kidwell et al. 1982) or can cause mutations due to imprecise excision, flanking DNA being excised in addition to the P-element itself (O'Hare and Rubin 1983). They can also have an influence on adjacent genes to the insertion site (Rubin and Spradling 1982).

The first work on the use of P-elements as gene transfer vectors was published in 1982 (Rubin and Spradling 1982; Spradling and Rubin 1982) and since then, they have been developed and refined into a powerful tool for genetic work in *Drosophila.* As previously

mentioned, engineered P-elements lack their own source of transposase and are manipulated in strains of flies that lack functional P-elements themselves (Ryder and Russell 2003; Castro and Carareto 2004). This enables the experimenter to control the provision of transposase via co-injection and genetic crossing. As mobilisation occurs through germline development pre-meiotically, mutations can then be recovered in the next generation.

1.7.3 Methods of Ectopic Gene Expression

Historically, a variety of methods have been used to ectopically express a gene of interest in *Drosophila* and these are described in more detail below.

Defined Promoter

One of the first methods employed was that of fusing the target gene of interest downstream of a characterised promoter (Basler, Christen et al. 1991). The use of promoters with tissue specificity enables transcription to be restricted to a specific subset of cells. There are distinct drawbacks to this approach. It is not easy to change the spatio-temporal expression pattern – this can only be achieved by fusing the gene of interest to a different promoter, which necessitates the cloning and characterisation of each promoter individually. In addition, expression levels are fairly inflexible – the only methods of varying them being via copy number or exploitation of position effect. It is also impossible

to establish and maintain lines where expression is toxic and lethality occurs (Zuker, Mismer et al. 1988; Parkhurst, Bopp et al. 1990; Parkhurst and Ish-Horowicz 1991).

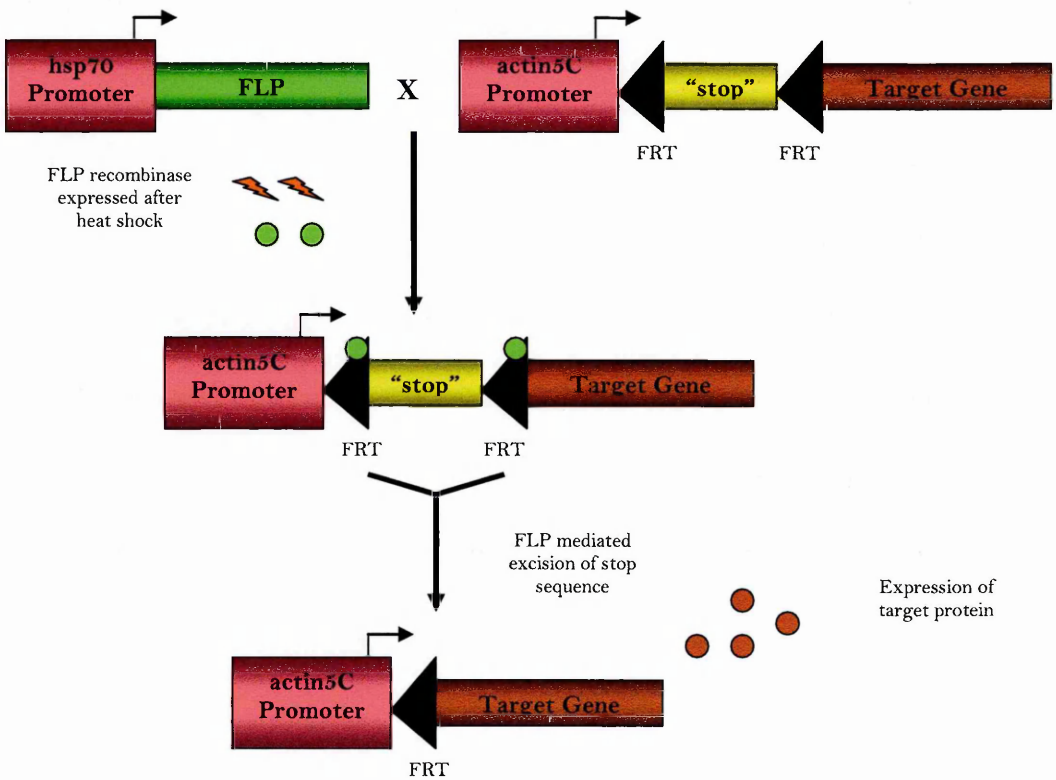
An extension of this technique is the use of a heatshock promoter to drive expression (Ish-Horowicz and Pinchin 1987; González-Reyes and Morata 1990). This has an advantage over the above method as it provides temporal control, dependent on the timing of the heatshock application. In addition, alteration of the temperature of the heatshock can affect the level of expression seen. However, this approach also has major drawbacks, the greatest of which is the 'all-or-nothing' status of heatshock gene expression. It is either off or on ubiquitously. Heatshock promoters are notoriously 'leaky', showing expression at varying levels without the application of heatshock. Heatshock delivery itself can induce phenocopies. This makes heatshock promoters unsuitable for lifespan studies. It is impossible to generate control lines that share a common, isogenised genetic background and that are also exposed to identical conditions. The 'leakiness' of the heatshock promoters mean that control lines may always show low levels of expression of the genes of interest. The application of daily heat stress to experimental lines means that comparisons between control lines, genetically identical but not exposed, are invalid.

FLP/FRT

The FLP/FRT system is a site specific recombination system which combines heatshock and characterised promoter methods and is described in Figure 1.7 (Sun and Tower 1999; Sun, Folk et al. 2002). Even though the effect of heatshock on control genotype lines has

been shown to have no positive effect on lifespan extension, with control lines showing no increase or a slight decrease in lifespan on exposure the heatshock (Sun and Tower 1999), there is no way to fully control for the possibly beneficial effects of the interaction between genes up- or downregulated by a heatshock pulse and the genes being expressed in the experimental lines. This is a major drawback of this expression system.

Figure 1.7 The FLP/FRT recombination system. Parental line where yeast FLP recombinase is expressed under heatshock control is crossed to a line containing the expression construct where the gene of interest is downstream of an actin5C promoter, separated by a transcriptional 'stop' construct. Resulting progeny are exposed to a brief heatshock which causes expression of FLP recombinase, which targets the FRT sequences flanking the 'stop' sequence, causing excision. This leads to constitutive expression of the target gene from the time of the heatshock pulse (Sun and Tower 1999).



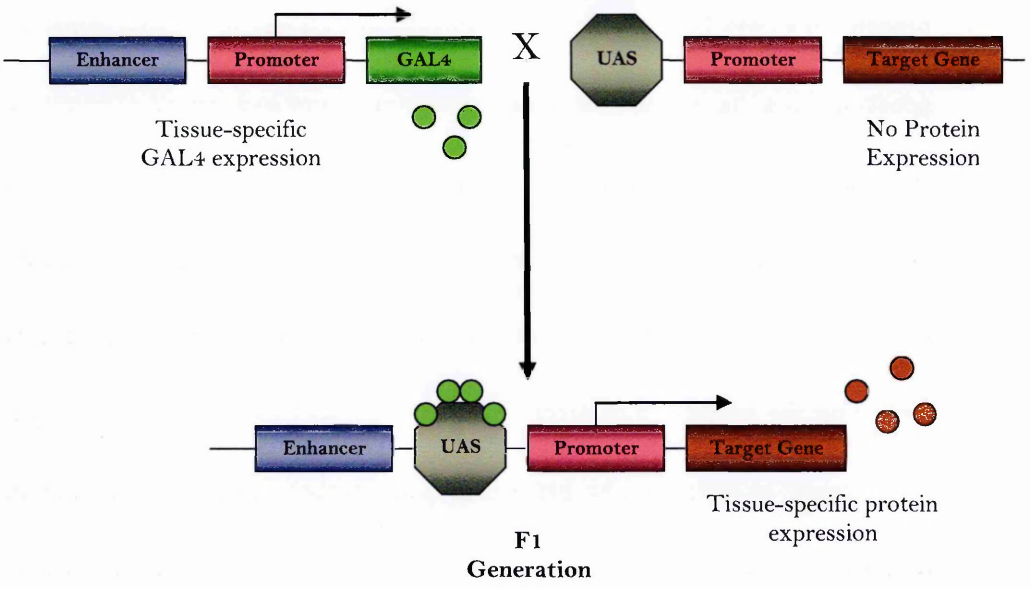
GAL4-UAS System

The GAL4-UAS system is a binary expression system that overcomes many of the problems with both heatshock and defined promoter methods of ectopic expression. It takes advantage of the binding and activation properties of GAL4, an 881-amino acid transcriptional activator found in the yeast *Saccharomyces cerevisiae* (Laughon and Gesteland 1984). GAL4 binds to four 17bp sequences located in the upstream activating sequence (UAS) of certain yeast genes (Fischer, Giniger et al. 1988) and these sequences have been demonstrated to activate transcription from promoters bearing the GAL4 binding sites in yeast (Giniger, Varnum et al. 1985), plants (Ma and Ptashne 1988), *Drosophila* (Fischer, Giniger et al. 1988) and mammals (Kakidani and Ptashne 1988; Webster, Jin et al. 1988; Ornitz, Moreadith et al. 1991).

Brand and Perrimon initially developed this system as a means of ectopic gene expression in *Drosophila melanogaster* (Brand and Perrimon 1993). This was achieved by fusing the GAL4 coding sequence to a P-transposase promoter. A vector was constructed which enabled the expression of GAL4 in a wide range of patterns and developmental stages, depending on the genomic site of integration, essentially removing the need for a variety of different promoters to be linked to the GAL4 gene (Brand and Perrimon 1993). One of the most commonly used vector plasmids is pUAST, into which the gene of interest is cloned (Hammond 2003). This includes a *Drosophila* promoter (Hsp70) linked to UAS enhancer repeats. The basic principle behind the system is illustrated in Figure 1.8. There are many advantages to this system. As the target gene (the UAS line) and the transcriptional activator (the GAL4 line) are both maintained as separate transgenic lines

with the gene remaining silent in the absence of the activator, it enables stable lines to be established for genes with toxic and lethal effects. The target gene is only activated in the progeny of a cross between the GAL4 and the UAS lines. It is a very efficient method as it generates lines that express the transcriptional activator in a variety of patterns. A library of these driver lines can then be used to express any target gene that is under UAS control in specific patterns. In addition, the expression patterns of the GAL4-driver lines can easily be verified in a non-invasive manner, using a variety of UAS-reporter lines where a gene regulating the expression of green fluorescent protein (GFP) is under UAS control. It is then simple to visualise the expression pattern throughout the whole life cycle of an individual fly (Phelps and Brand 1998). GAL4 does not appear to have any deleterious effects in *Drosophila* (Duffy 2002) although when using this method for lifespan assays, it is still necessary to control for its presence. The ability to remobilise the P-element containing the gene under UAS control by crossing to lines containing a source of transposase means that it is relatively simple to generate a number of insertion lines and hence control for the effects of the position of insertion.

Figure 1.8 The GAL4-UAS system. A GAL4 driver line expressing the yeast transcriptional activator in a tissue-specific manner is crossed to a UAS-responder line. In the progeny of this cross, GAL4 binds to the upstream activating sequence driving target gene expression in a tissue dependent pattern (Brand and Perrimon 1993).



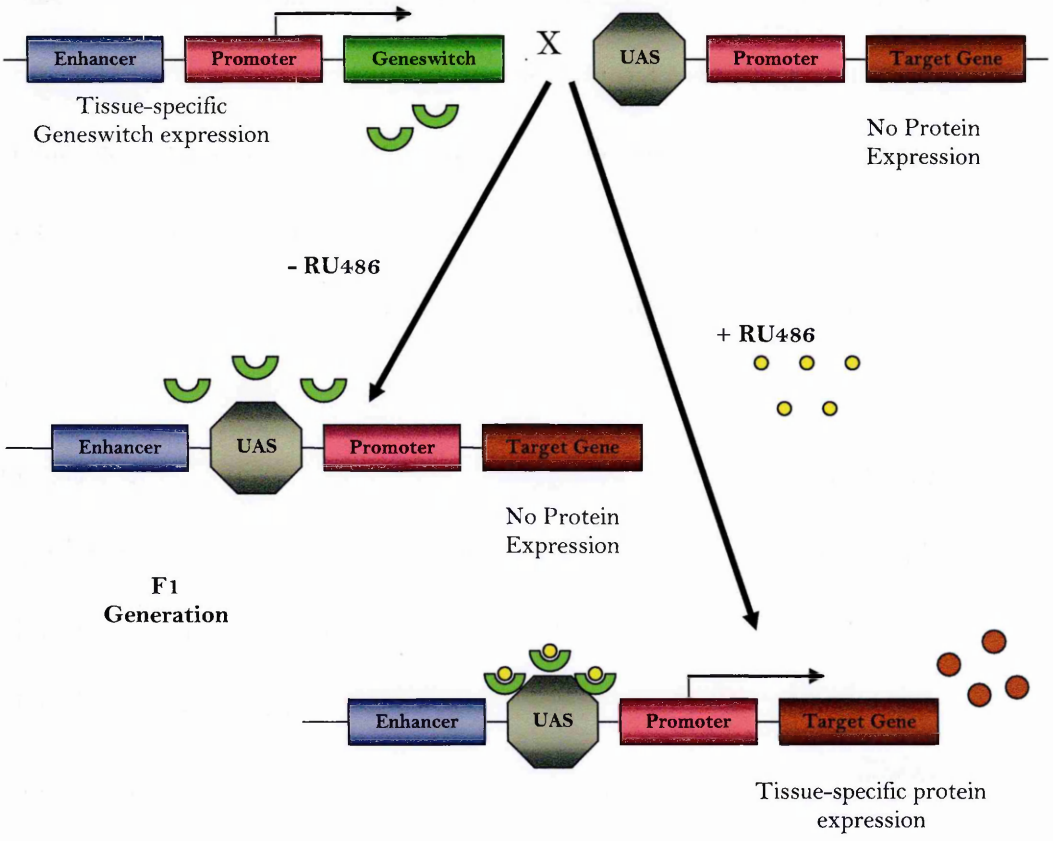
There are certain drawbacks to this system. Driver lines are not always specific solely to one type of tissue. It is a common problem that drivers express in the tissue of interest and also in the salivary glands of the fly (Brand and Perrimon 1993). This is believed to be as a result of the presence of a salivary gland enhancer in the hsp70 sequences upstream of the GAL4 coding region (Gerlitz, Nellen et al. 2002). Elimination of the 5' UTR sequences did remove this salivary gland expression, but at the cost of a reduction in the function as enhancer trap vectors (Gerlitz, Nellen et al. 2002). This is not necessarily problematic when using the drivers, but is certainly something that has to be borne in mind when analysing the effects of gene overexpression. There is also some distortion of temporal control of expression by GAL4. Transcription of the UAS-target gene shows a degree of lag behind the start of promoter GAL4-transcription as a certain GAL4 level is required before the target gene is activated (Phelps and Brand 1998). Also, GAL4 may perdure, which means that some protein will be present and driving UAS gene expression after promoter-

GAL4 transcription has ended (Phelps and Brand 1998). It should also be noted that GAL4 expression is temperature dependent with minimal expression at 16°. The optimal temperature, balancing maximal expression and fertility/viability, is 29°, something which needs to be taken into account when running experiments (Duffy 2002). A wide range of expression levels of the target gene can be achieved by varying the temperature within this range (Duffy 2002). In addition, as expression in progeny is achieved by crossing two parental lines, generating relevant control genotypes is not a simple issue. Genetic background remains an issue and it means that it is necessary to isogenise any lines that will be used in the generation of experimental and control genotypes so that the only difference is the expression/silence of the gene of interest. Whilst this provides a tissue specific expression system, temporal control is still poor. There are drivers which express at certain times during development but it is not possible, for example, to express and turn off a gene at will.

Geneswitch System

This method of ectopic gene expression, based on the GAL4-UAS system but using a GAL4-progesterone receptor fusion protein (Geneswitch) whose transcriptional activity depends on the presence of the progesterone hormone RU486 (mifepristone), enables much greater spatiotemporal control than any of the previously described methods (Osterwalder, Yoon et al. 2001; Roman, Endo et al. 2001).

Figure 1.9 The Geneswitch system. Geneswitch driver line expressing GAL4-progesterone receptor fusion protein in a tissue-specific pattern is crossed to a UAS-responder line carrying the gene of interest. In the absence of RU486 in the food media, the Geneswitch fusion protein cannot bind to the UAS sequence. In the presence of RU486, the Geneswitch fusion protein binds to the UAS sequence driving target gene expression in a tissue-specific pattern (Osterwalder, Yoon et al. 2001)



The principle behind the system is illustrated in Figure 1.9. The key difference between this mode of expression and the GAL4-UAS system is that the progeny of GeneSwitch-UAS parent cross express the fusion protein (GeneSwitch) instead of GAL4, which can only bind to the UAS element in the presence of RU486. This provides a means of controlling the temporal expression of the gene of interest by supplementing the food media with RU486. Osterwalder and colleagues (2001) used this method to express GeneSwitch in muscles and neurons by using cloned promoter fragments of the *embryonic lethal abnormal vision* (*elav*) gene and the *myosin heavy chain* (*MHC*) gene (Osterwalder,

Yoon et al. 2001) This temporal control (including the ability to turn off expression at any time by withholding RU486 from the food media) is a major advantage of the system. In addition, it provides a good control for genetic background – genetically identical sibling flies can be used, with expression controlled entirely by dietary supplementation. In lifespan assays extra controls should be set up to control for the presence of GAL4 and the effect of RU486 on the animals but no adverse effects of GAL4 on the physiology and lifespan of flies have been shown (Duffy 2002).

Certain characteristics of this system should be noted, although they are not necessarily drawbacks. There is an observed time-lag between RU486 feeding and reporter protein expression, with a possible delay of up to 5 hours between feeding and target gene expression (Osterwalder, Yoon et al. 2001). Transgene expression is dose-dependent on RU486 concentration (Osterwalder, Yoon et al. 2001; Poirier, Shane et al. 2008). More importantly, levels of transgene expression have been shown to vary according to the age, sex and strain of animal (Poirier, Shane et al. 2008). This has wide-reaching implications for the analysis of lifespan studies. In adult animals RU486 is delivered via oral ingestion in the food media, therefore transgene expression could be reduced as an animal ages and feeds less (Carey, Papadopoulos et al. 2006). Recent analysis of the spatial expression patterns of several driver strains showed less tissue specificity than initially published (Poirier, Shane et al. 2008). Most importantly, a low level of background expression in the absence of the inducer has been recorded (Osterwalder, Yoon et al. 2001) and this has been characterised in detail for various drivers (Poirier, Shane et al. 2008).

Despite these issues, the GeneSwitch system still remains the most suitable system for the study of gene expression and its effects on lifespan. Careful note of all of the above should be taken, however, when interpreting results from lifespan studies using this method.

1.7.4 Range of Assays

As mentioned above in Section 1.7.1, one of the advantages of *Drosophila melanongaster* as a model system for the study of ageing and stress resistance is the range of assays available to the researcher.

Lifespan studies are an example of the flexibility this organism offers. The short lifespan of flies and their prolific breeding rate in relation to other laboratory animals such as rodents enables these studies to be carried out with a high sample size and a good replication number, enabling powerful statistical analyses to be carried out. Although there are issues with genetic background in *Drosophila* (see Section 1.7.5), these can be overcome by choosing a suitable expression system (see Section 1.7.3). Section 1.8 contains a more detailed examination of different lifespan studies in *Drosophila.*

It is also possible to expose flies to a variety of chemical stressors in order to examine their response to oxidative stress. One of the most commonly used stressors in *Drosophila* is paraquat (1,1'-dimethyl-4,4'-bi-pyridinium). It creates oxidative stress via the production of $O_2^{\bullet -}$, a product of the NADPH-dependent reduction of Pq^{2+} to the relatively stable Pq^{+}

radical which readily reacts with O_2 producing $O_2^{\bullet -}$ (Hassan and Fridovich 1978; Seto, Hayashi et al. 1990). Flies selected for resistance to paraquat also show an extended longevity phenotype (Vettraino, Buck et al. 2001) and it has been widely used as an assay to examine the effect of manipulation of genes involved in antioxidant defence (Orr and Sohal 1993; Mockett, Orr et al. 2001; Duttaroy, Paul et al. 2003).

Diethyl maleate is a compound which acts to deplete glutathione itself rather than functioning as an oxidative stressor. The electrophilic nature of its centre facilitates reaction with glutathione directly or via the glutathione-S-transferase system (Boyland and Chasseaud 1967; Bannai 1984), therefore it functions as a glutathione depletor rather than an oxidative stressor. It is likely, however, that a depletion of glutathione will lead to a more oxidising cellular environment, hence increasing the organismal oxidative stress level. DEM has been effectively used to deplete glutathione levels in human cell culture (Ruiz, Siow et al. 2002; Szaszi, Jones et al. 2005; Kim, Barajas et al. 2007) and when administered via intraperitoneal injection in mice (Kaur, Kalia et al. 2006).

In addition to these chemicals, a more oxidising environment can be created by exposing flies to H_2O_2 or keeping them in a hyperoxic environment with atmosphere composed of 100% O_2 (Mockett, Orr et al. 1999; Mockett, Orr et al. 2001). These are both more complicated assays to carry out, with H_2O_2 being less stable than either paraquat or DEM at room temperature. The delivery of O_2 necessitates the raising of fly cultures in oxygen chambers. Hyperoxia has been criticised as an effective mode for accelerated ageing as

redox status shifts seen in normal ageing are not replicated under hyperoxic conditions (Rebrin and Sohal, 2006). It is also possible to expose flies to ionising radiation in order to examine its physiological effects in relation to the oxidative defence system (Parkes, Kirby et al. 1998). A variety of physical stressors can easily be applied to flies, in particular, heat stress, cold stress, starvation and desiccation (Mockett, Orr et al. 2001).

In addition, there is potential to study physiological factors. It is possible to study olfactory and spatial memory in *Drosophila* (Berry, Krause et al. 2008; Masek and Heisenberg 2008; Neuser, Triphan et al. 2008; Wang, Mamiya et al. 2008) and there are a variety of approaches to studying flight at the level of individual muscles (Elliott, Brunger et al. 2007) and whole fly flight performance (Valente, Golani et al. 2007; Grover, Tower et al. 2008) making it possible to examine changes in physical and mental ability as a flies age (Miller, Lekkas et al. 2008).

All of these are reasons why *Drosophila melanogaster* is a versatile and practical model organism in which to carry out lifespan and oxidative stress investigations.

1.7.5 Confounding Factors in Lifespan Analysis in *Drosophila melanogaster*

When designing lifespan experiments, there are several confounding factors which need to be addressed in the experimental design, the most important of which are dealt with below.

Genetic Background

It is vital in lifespan studies focusing on individual genes to be able to ensure that the expression of the particular gene in question is responsible for any extension seen. This is problematic as the genetic background of each experimental and control line could be remarkably diverse. The presence of mutations or P-elements elsewhere in the genome and unrelated to the gene of interest could have profound implications for the lifespan of the organism. Mitochondrial genotype has a strong effect on lifespan, possibly modulated by nuclear genotype (Clancy 2008). A retrospective analysis of a number of lifespan studies involving antioxidant enzymes has exposed a negative correlation between control lifespan and lifespan increase, with the greatest extension seen in lines where the relevant control lifespan is the shortest (Orr and Sohal 2003). In a separate study, expression of those antioxidants in flies with a long-lived background leads to much more modest lifespan increases or none at all and effects were highly correlated with genetic background (Spencer, Howell et al. 2003). Specific allelic differences in the gene *methuselah* lead to differing lifespans (Paaby and Schmidt 2008) and locomotor activity in ageing flies depends, in part, on the genetic background of the fly strain assessed (Fernandez, Grant et al. 1999). Although there are approaches which can minimise the variation in genetic background between strains (repeated outcrossing to a single reference strain, sibling mating or the use of balancer chromosomes to effect a complete chromosome replacement), it is far better to use genetically identical lines and overexpress solely the gene of interest. This can be achieved by using the GeneSwitch system (Section 1.7.3). The original lifespan of the control lines still needs to be taken into account but it minimises the effect of background between control and experimental lines.

Culture Conditions

Larval population density is known to affect lifespan (Buck, Nicholson et al. 1993) and other morphological traits in *Drosophila* (Imasheva and Bubliy 2003). It is necessary to take this into account when designing lifespan studies, raising larvae at equal, low population densities for experimental and control genotypes. In addition, temperature and stress effects on larvae can have an effect on lifespan. It is necessary, therefore, to ensure that conditions are kept as uniform as possible during the generation of flies for lifespan assays and that both control and experimental lines are kept in the same environment.

1.7.6 Characterising the Ageing Phenotype in *Drosophila melanogaster*

Much work has been done to characterise the ageing phenotype in *Drosophila melanogaster.* The advent of microarray technology has enabled large scale transcriptional studies to be carried out on aged vs young flies, widening the picture of transcriptional changes that occur with age. Sohal and Weindruch measured transcriptional changes in aged vs young flies under normal nutritional conditions and conditions of caloric restriction, finding 885 age-related genes (Sohal and Weindruch 1996). Work by Landis *et al* supported the oxidative stress theory of ageing, comparing transcriptional profiles of flies raised in hyperoxic conditions with those of aged flies and finding upregulation of several similar pathways (Landis, Abdueva et al. 2004). Studies using both cDNA microarrays and Affymetrix chips have demonstrated differing profiles in different tissues, suggesting that

ageing affects each tissue type in an individual way (Kim, Rhee et al. 2005; Girardot, Lasbleiz et al. 2006).

There are many physiological changes that have been examined in *Drosophila.* Aged flies have been shown to have impaired flight ability resulting from structural deterioration and mitochondrial damage within the flight muscle. In addition, Ca^{2+} activation of some muscle fibres fails with age (Miller, Lekkas et al. 2008). Taking advantage of the fact that *Drosophila* exhibit negative geotaxis (i.e. a tendency to move in the opposite direction to the force of gravity), Mockett *et al* showed that flies from long-lived lines showed a slower age-related decrease in walking speed than short-lived flies (Mockett, Orr et al. 2001). However, work by Fernandez *et al* suggests that using decreased locomotor activity as a biomarker of ageing must be treated with caution as although they found that mean locomotor activity decreases with age across the strains they studied, this decrease was not observed in all strains of flies and could be affected by genetic background and sex of flies (Fernandez, Grant et al. 1999). Ageing *Drosophila* also develop an acquired susceptibility to temperature-sensitive paralysis and this has been proposed as a useful biomarker of ageing (Reenan and Rogina 2008). Different populations of stem cells are affected in different ways during ageing, with germline stem cells showing a reduced division rate (Zhao, Xuan et al. 2008) and intestinal stem cells and progenitor cells showing an age-related increase in activity (Choi, Kim et al. 2008). This is interesting from the perspective of investigations into the link between stem cells and cancer. Immune response is also compromised in ageing *Drosophila,* with individuals showing reduced capacity to survive bacterial infection (Ramsden, Cheung et al. 2008). Much like humans, *Drosophila* develop

alterations in sleep duration and pattern as they age (Shaw, Cirelli et al. 2000; Koh, Evans et al. 2006). Flies also show a deterioration in the regularity of their heartbeat, with arrythmias occurring more frequently (Ocorr, Reeves et al. 2007) although organismal survival is less closely linked to heart function than in vertebrates (Ocorr, Perrin et al. 2007).

Despite the above studies, there is still relatively little consensus about which biochemical and physiological states are reliable biomarkers of ageing. Confounding factors such as genetic background and epigenetic interactions mean that it is not often clear how a certain trait will alter as a fly ages. Underlying the gross physical changes such as locomotor activity and flight, are changes at a more complex level, involving the biochemistry and physiology of individual components of flies' anatomy such as motorneurons, responsible for movement and flight. A case has been made that motorneurons are the ultimate limiting tissue in determining fly lifespan and this will be examined in more detail in Section 1.8.2 and in Chapter 4.

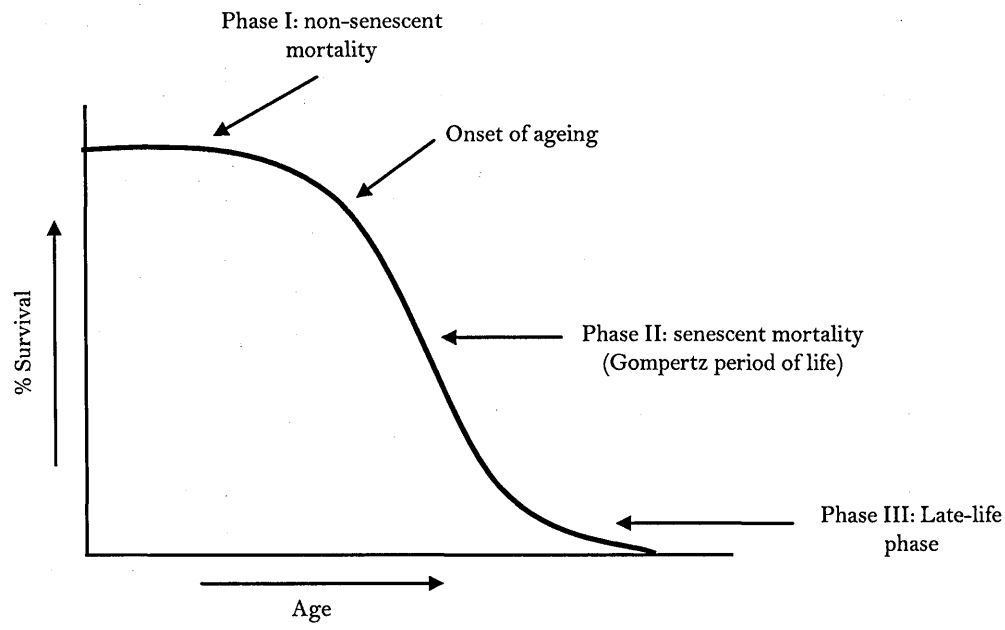
1.8. Lifespan Studies in *Drosophila melanogaster*

A large body of work has already been amassed investigating the effects of a vast array of conditions on lifespan in *Drosophila.* It can be subdivided into several main areas: in particular, manipulations involving the antioxidant defence system, those concerned with caloric restriction and the insulin/IGF signalling pathway and a variety of other genes and treatments which have been shown to affect longevity. This section will focus on the effect of the manipulation of a variety of antioxidant enzymes lifespan in *Drosophila.* It will also briefly review other genetic interventions and treatments that have been demonstrated to increase longevity. Although a detailed treatment of each topic is not possible within the constraints of this review, it should be noted that the volume and breadth of treatments and genetic interventions that have been shown to extend lifespan in *Drosophila* exemplify the complex, multifactorial nature of the ageing process in the fruitfly.

1.8.1 The Lifespan Curve – Dissecting Longevity Curve Profiles

Survival curves have a distinctive morphology that can be divided into three main phases. It is necessary to clarify these phases as they have been referred to in the results chapters of this thesis. Figure 2.3 shows a typical survival curve with the relevant sections highlighted and the terminology that has been used later in this thesis is shown.

Figure 1.10 Schematic of a typical lifespan curve showing the main phases



Benjamin Gompertz originally characterised the exponential growth of mortality in 1825. It is the significant deviation from this exponential increase that is seen in early and later life that gives rise to the curve profile shown in Figure 1.10 (Doubal and Klemera 1999).

For the purpose of clarity and uniformity in this thesis, 3 distinct phases in the mortality curve have been distinguished. Phase I refers to the initial early-life plateau phase of the lifespan curve where the risk of mortality is age-independent, i.e. the risk comprises non-age related factors and does not increase exponentially with age. Mortality in this phase of the curve is indicative of early life events/conditions which have an effect on survival rather than any ageing-related conditions (Johnson, McKechnie et al. 2006). This could encompass developmental impairment that gives rise to early post eclosion mortality or external conditions such as adverse reaction to collection, handling and anaesthesia. Phase

II, senescent mortality, refers to the so-called Gompertz period of life (Doubal and Klemera 1999). During this phase, the risk of mortality is age-dependent and increases exponentially with age. The slope of the curve can provide an estimation of the mortality rate – the steeper the curve, the higher the rate of mortality. Mortality during this phase is likely to comprise both effects from early life events that cause later life mortality differences and mortality due to age-related conditions. Phase III refers to the late-life period where the curve deviates from the Gompertz model of exponential mortality increase. Two reasons have been suggested for this late-life reduction in mortality: that individuals with a greater risk of death have gradually decreased within the population and it comprises more 'age-resistant' individuals or that there are changes in the fundamental mechanisms of ageing in an older population (Doubal and Klemera 1999).

In addition to the three phases described above, the point where the slope change is maximal on the curve (see Figure 1.10) is taken as the time of onset of ageing. There is no standard analytical method for determining this point (Johnson, McKechnie et al. 2006). Although these points of maximal acceleration in hazard rates and hence presumed onset of age-related damage accumulation have been calculated for large-scale human studies (Luder 1993), the reduced amount of data points generated by smaller scale lifespan studies in the laboratory means that this computation is not valid for the data analysed in this thesis. Semi-analytic methods have been proposed (Johnson, McKechnie et al. 2006), however, this provides little real benefit over assessing by eye the point of maximal change of the curve slope. For the purpose of this thesis, the onset of ageing, defined as the point of maximal slope change, has been assessed by eye on each curve.

1.8.2 SOD and Catalase: Their Role in Lifespan Extension

The interaction between SOD, lifespan and stress resistance has been examined using two main approaches *in vivo*: reduction and overexpression of different types of SOD.

Under-expression Studies

Initially, this section will focus on studies that have centred on the reduction of SOD levels in *Drosophila*. An in-depth analysis of this is beyond the scope of this thesis as reduction of the level of certain enzymes does little to elucidate their role in ageing. Reduction is an extreme perturbation from biological state of 'normal' and, therefore, it would be expected that this should reduce longevity – over-expression studies which are accompanied by lifespan extension are much more informative from the perspective of examining a gene's role in ageing. Therefore, despite reduction in SOD levels producing phenotypes which are subsequently rescued by a return to normal levels, this cannot be used to support its role in lifespan extension, it only indicates that removal of SOD impairs lifespan and restoration rescues that impairment. Studies where SOD expression has been reduced/removed are summarised below in Table 1.3. An important point to be noted from these studies is the specific sensitivity to paraquat and other oxidative stressors that these lines demonstrate. Although none of the papers included assays using a toxin whose action was not related to the oxidative defence system meaning that there were no controls for a general 'poisoning' effect in fly lines which could purely be less robust, it appears that lines with impaired global SOD gene function no longer handle oxidative insult efficiently. It should be noted that genetic background was only addressed in one study (Parkes, Kirby et al. 1998).

Table 1.3: Review of the phenotypic effects of a reduction in SOD in *Drosophila melanogaster*

Paper Ref	Fly Stocks	Description	Type of SOD	Phenotype
(Phillips, Campbell et al. 1989)	$cSod^{n108}$	EMS-induced recessive lethal null mutation	Cu/ZnSOD	Hypersensitivity to paraquat, Cu(I); adult longevity 76-81% reduction
(Parkes, Kirby et al. 1998)	transgene$^-$; $cSod^{n108}$ transgene$^+$; $cSod^{n108}$	EMS-induced recessive lethal null mutation in background *Drosophila* cSOD genomic sequence under control of native promoter (+/- transgene)	Cu/ZnSOD	Comparable genetic backgrounds; extreme sensitivity to paraquat, sterility, reduced lifespan hypersensitivity to ionising radiation, hypersensitivity to glutathione depletion; all rescued in transgene$^+$ background
(Kirby, Hu et al. 2002)	SodIR15 SodIR24	GAL4-regulated inverted repeat SOD2 RNAi transgene Eliminates detectable SOD2	MnSOD	Hypersensitivity to paraquat; no major impact on pre-adult viability; rapid onset adult mortality (reduction 86% & 76%)
(Duttaroy, Paul et al. 2003)	$Sod2^{n283}$	Strong loss of function allele (generated by P-element KG06854 excision)	MnSOD	Impaired longevity Hypersensitivity to paraquat
(Woodruff, Phillips et al. 2004)	Sod^{n108}	EMS-induced recessive lethal null mutation	Cu/ZnSOD	Small but significant effect on germline mutation rate Rate of genomic damage leading to mutation &/or recombination during somatic development extensive Extensive but normally repairable damage to DNA, in repair deficient background, near lethal and extensive damage
(Paul, Belton et al. 2007)	$Sod2^{n283}$ $Sod2^{wk/wk}$ KG06854R	Strong loss of function allele (generated by P-element KG06854 excision) Reduction of expression (P-element insertion (KG06854) in Sod2 5'-UTR) Revertant control derived from precise excision of KG06854	MnSOD	Mean/max lifespan reduced by 20%-24% with 50% SOD2 activity and 38-43% at 75% reduction of SOD2 activity) Increased slope of mortality plot not changes in initial mortality rate Oxidative damage to acontinase, DNA (causing strand breakage) Age related decline in olfactory behaviour

Overexpression Studies

The story regarding the relationship between SOD overexpression and lifespan is a complex one and serves as a cautionary tale with regards to the effect of genetic background in this kind of study. As discussed in Section 1.4.2, SOD is a key component in the antioxidant defence system. The oxidative damage theory of ageing proposes that an accumulation of age-related oxidative damage is a defining factor in the lifespan and manner in which organisms age. Based on this, it can be hypothesised that an increase in certain components of the oxidative defence system in *Drosophila* will be beneficial. This question has been quite comprehensively investigated in the case of SOD, from the perspective of both lifespan and stress resistance.

Ubiquitous Overexpression Studies

Cu/ZnSOD and MnSOD have been overexpressed successfully in *Drosophila*, both alone and in conjunction with catalase. In studies where Cu/ZnSOD has been expressed alone, it has been shown to have little benefit to lifespan (Orr and Sohal 1993; Sun and Tower 1999). In Orr and Sohal's study, *Drosophila* SOD was overexpressed resulting in increases of between 32-42% and yet only a minor, insignificant increase in lifespan was seen (Orr and Sohal 1993). Flies which showed a small increase in mean lifespan did also show an increase in resistance to hyperoxia but not to paraquat, implying that the relationship between lifespan extension and stress resistance is far from straightforward. Sun and Tower, whilst reporting extension in some lines overexpressing solely Cu/ZnSOD, show that it is highly dependent on line and genetic background (Sun and Tower 1999).

MnSOD overexpression, in the absence of a concurrent overexpression of catalase, actually led to a decrease in lifespan (Mockett, Orr et al. 1999). In addition, there was no difference in protein carbonyl content (an indicator of oxidative damage) and no increased resistance to 100% O_2 (Mockett, Orr et al. 1999), suggesting that the lifespan decrease may be unconnected to the antioxidant status within these lines.

When Cu/ZnSOD was expressed in combination with catalase, providing less of an imbalance in the SOD/catalase pathway, significant lifespan extension of up to 34% in relation to control lines was seen (Sohal, Agarwal et al. 1995). In addition, there were significant reductions in oxidative damage products and 8-OHdG content and a delayed loss of physical vigour (Sohal, Agarwal et al. 1995). However, a later study specifically concentrating on determining whether these life-extending effects persisted in flies with a long-lived background found no beneficial effect on survivorship of the overexpression of Cu/ZnSOD and catalase in long-lived lines (Orr, Mockett et al. 2003). This has led to the conclusion that increases in the activities of SOD and catalase does not decrease the rate of ageing in long-lived strains, only having an effect in strains with a less robust genetic background (Orr, Mockett et al. 2003; Orr and Sohal 2003). This further implies that antioxidant levels, at least globally in an organism, are not the defining factor in the balance between oxidant production, antioxidant defences and repair processes (Orr, Mockett et al. 2003). Interestingly, one study reports that overexpression of Cu/ZnSOD throughout development results in pupal lethality and high levels of lipofuscin, an age-related peroxidation end-product that is symptomatic of membrane damage and mitochondrial

damage (Seto, Hayashi et al. 1990), implying that the consequences of overexpression are not necessarily neutral in *Drosophila.*

Overexpression of MnSOD in conjunction with catalase has no effect on lifespan and flies demonstrate no compensatory metabolic changes, no effect on their physical activity levels and no change in the levels of other antioxidants (Mockett, Bayne et al. 2003; Bayne, Mockett et al. 2005). These fly lines did, however, show an increase in resistance to H_2O_2, paraquat and cold stress (Mockett, Bayne et al. 2003; Bayne, Mockett et al. 2005). Resistance to hyperoxia was variable with one study reporting an increase (Mockett, Bayne et al. 2003) and another a decrease (Bayne, Mockett et al. 2005).

Tissue-Targeted Overexpression Studies

As no firm evidence exists, that global SOD and catalase levels are the defining factor in lifespan and age-related decline in *Drosophila*, the search for the key to lifespan extension in flies has moved to more specific, tissue-targeted models. The most important of these to be examined so far, is the role that motorneurons and antioxidants play in lifespan and stress resistance in flies.

In 1998, Parkes *et al* overexpressed human Cu/ZnSOD (SOD1) in *Drosophila* using the GAL4-UAS system and a motor neuron specific driver (*D42-GAL4*) (see Section 1.7.3) and observed lifespan extension of up to 40% in their experimental lines (Parkes, Elia et al. 1998). This was mainly due to an extension of the pre-mortality plateau stage (see Figure

1.10) from 27 days to approximately 50 days and was accompanied by enhanced resistance to paraquat (Parkes, Elia et al. 1998). This has been cited as evidence that lifespan is determined by reactive oxygen metabolism in critical cell types included in the motorneuron (Parkes, Hilliker et al. 1999). Parkes goes further in arguing that, in the absence of factors such as predation and disease, the lifespan of wild-type *Drosophila* is regulated by reactive oxygen-mediated failure of motorneurons (Parkes, Hilliker et al. 1999). There are several key flaws with these papers that invalidate these statements. It must be noted that in his 1998 study, Parkes overexpressed the human form of Cu/ZnSOD (SOD1) in *Drosophila* motorneurons rather than *Drosophila* Cu/ZnSOD, which may have had an effect on the results. More importantly, expression was verified by *in situ* hybridisation, focused on tissues containing motorneurons. The D42-GAL4 driver that was used is also expressed very strongly in the salivary glands of the fly (see Chapter 4). This is a known issue with GAL4 drivers and was addressed in Section 1.7.3. This means that the western blots and SOD activity assays that this paper cites, which were based on whole fly preps, do not accurately represent levels in the motorneurons. This is an important issue as Parkes goes on to argue that the total amount of SOD1 produced by D42-GAL4 activation approximates that produced throughout the body of a wild-type fly, using this as support for the statement that the level of SOD1 in motorneurons of the overexpressing flies is orders of magnitude higher than normal levels, suggesting that intervention in the intracellular signalling role of SOD may play a part in the mechanism of lifespan extension seen here and that that can only be achieved at extremely high levels (Parkes, Hilliker et al. 1999). In fact, it is impossible to conclude that from these data. Certainly the level of SOD in motorneurons is low under normal circumstances (Klichko,

Radyuk et al. 1999) but no conclusions can be drawn from these data with regards to the levels achieved through overexpression using this driver. In addition, the low levels of SOD present ordinarily in the motorneurons means that Parkes' study overexpresses something in an environment where it is not normally present and it is difficult to conclude what role SOD1 plays in the normal course of ageing under these circumstances. It has been argued that the low level of SOD in motorneurons is the limiting factor in lifespan and age-related decline (Phillips, Parkes et al. 2000). Although this is possible, it is impossible to say with certainty without assessing the actual damage to motorneurons; elevated levels alone do not provide enough evidence, especially as there are contradictory results showing that locomotor activity and flight performance are not consistently reduced in a non-line-specific manner as flies age, processes which depend on good motorneuron function (Fernandez, Grant et al. 1999). Neither, however, do these studies preclude the possibility of motorneuron function playing a key role in the ageing process. The next step in this investigation is to examine the effects of other antioxidant enzymes in this and other tissue-specific patterns, as this thesis does, bearing in mind the issues raised by these papers.

1.8.3 The Relationship Between Glutathione and Lifespan Extension

Recently, two papers were published that systematically investigated the effects of the overexpression of the individual subunits of GCL in the nervous system of the fruitfly. The results from these papers are summarised in Table 1.4. These results will be discussed in detail in the context of the results presented in this thesis in Chapters 3-5, but there are

certain key points that should be noted. These results strongly support the prediction that the specific tissue where overexpression occurs has a strong effect on lifespan phenotype. This work goes further than the studies described in Section 1.8.2, using a variety of GAL4 drivers that express in specific neuronal tissues.

For the catalytic subunit alone, overexpression globally and in neural regions leads to an increase in glutathione content, assayed in whole body extract and specifically in heads for the neural drivers. However, significant and reproducible lifespan extension was only seen when GCLC was overexpressed in motor neurons and the mushroom bodies (Orr, Radyuk et al. 2005; Luchak, Prabhudesai et al. 2007). Pan-neural overexpression provided less consistent increases in lifespan. This result is consistent with the hypothesis that an optimum level of antioxidant expression exists for individual tissue types. It is possible that the positive effect of overexpression in mushroom bodies is diminished by expression in a pan-neural pattern, where negative or neutral effects in the rest of the neural tissue counteract the positive effects in this specific regions. The fact that *elav-GAL4* and *Apple-GAL4* driven expression have variable results between individual lines implies that position effect may play a role in the extension seen. Global overexpression was assessed using two different drivers: *Tub-GAL4* drove expression at very high levels (4-6 fold increase in enzyme levels and 85-104% in total glutathione) whilst *armadillo-GAL4* drove global overexpression at a more modest level (1.5-2.0 fold increase) (Orr, Radyuk et al. 2005; Luchak, Prabhudesai et al. 2007). Any lifespan benefits accrued as a result of overexpression of GCLC were shown to be dependent on expression levels; low level global overexpression giving rise to a significant increase not seen at higher levels. This expression

level-dependent effect further supports the suggestion of an optimum level of expression in certain tissues, with negative effects of high-level overexpression counteracting any increases seen from targeted neuronal overexpression. The effects on lifespan appear to be dose-dependent as lower levels of global expression are, in fact, beneficial. No difference is seen in oxygen consumption levels, suggesting that the extended longevity phenotypes were not a result of any compensatory reduction in metabolic activity (Orr, Radyuk et al. 2005; Luchak, Prabhudesai et al. 2007).

The relationship between lifespan and stress resistance is again complicated, with no effect in young (10 day old) flies exposed to H_2O_2 and paraquat when overexpression was driven in the motorneurons or in a pan-neural pattern but significant extension when assays were repeated using 40 day old flies (Orr, Radyuk et al. 2005). It must be noted that these stressors are applied in a non-targeted global manner by delivery in food media. The implication of these data is that as a fly ages, there is a shift in the profile of tissues which are lifespan-limiting under conditions of acute oxidative stress. In young flies, overexpression in neural regions has no effect on survival, indicating that stress response in this tissue is not the limiting factor. However, as the flies age, overexpression in the same tissues does lead to extension of survival time and therefore these must become limiting tissues. It is possible that young flies already have an optimum level of expression of components of the antioxidant defence system and that this balance may shift as a fly ages. The question remains as to whether there is a specific decline in stress response in neural tissues or whether the low levels of expression in these tissues *in vivo* mean that these tissues are purely more sensitive to a global decline in antioxidant defence. An alternative

explanation is that other tissues affected by the global application of stress play a more important role in younger flies than neural tissue. As increased expression is only in the neural tissue, these alternate tissues are afforded no extra protection from the increased stress levels. Neither paper includes data for global overexpression at high or low levels.

Overexpression of the modifier subunit, GCLM, in any of the neural tissues provides no significant increase in lifespan. However, global overexpression leading to a 25-50% increase in total glutathione content gives a significant increase in mean lifespan of 24% (Orr, Radyuk et al. 2005). This is comparable with increases in lifespan seen with low-level *armadillo-GAL4* driven GCLC expression and supports the dose-dependent nature of lifespan extension. No data are shown in this study to indicate levels of glutathione or enzyme expression in the heads of flies overexpressing GCLM.

Putative mechanisms behind the lifespan extension shown by these authors will be discussed in detail in Chapter 5 in relation to the results presented in this thesis, however, certain aspects of the design of these studies should be considered. The two papers cited here use multiple insertion lines which provides a good control for position effects of transgene insertions. The control lines are also described as being relatively long-lived and an attempt was made to isogenise the lines by backcrossing. However, a major drawback is that the GAL4 drivers used are expressed throughout development which makes it impossible to dissociate the developmental effects of manipulation of glutathione titres from the effects in adult flies. This is important as there are developmental effects resulting

in lethality when both sub-units are co-overexpressed in a recombinant fly at high levels, as discussed in more detail in Chapter 3. This thesis attempts to further elucidate the role of glutathione in lifespan and stress resistance, taking advantage of the GeneSwitch system (Section 1.7.3) to bypass the developmental effects which will underlie the data described here.

Table 1.4: Review of the phenotypic effects of overexpression of the catalytic (GCLC) and modifier (GCLM) subunits of GCL in different tissues in *Drosophila melanogaster*

Paper Ref	Subunit	Driver/Tissue	Expression Increase	Extension Reported
(Orr, Radyuk et al. 2005)	GCLC	*Tub-GAL4* (high-level global)	4-6 fold (enzyme assay/immunoblot) 85-104% (GSH content)	No significant increase
		D42-GAL4 (motor neuron)	Slight in whole body (enzyme assay/immunoblot) Significant in heads (enzyme assay/immunoblot)	Significant & reproducible increase in mean and max
		elav-GAL4 (pan-neural)	Slight in whole body (enzyme assay/immunoblot) Significant in heads (enzyme assay/immunoblot 10-23% (GSH content)	Positive effects but only significant and reproducible in 1 line
		Appl-GAL4 (pan-neural)	Slight in whole body (enzyme assay/immunoblot) 70-118% in heads vs 16-29% in thoraces (GSH content)	Significant and reproducible increase in 2 out of 4 lines
(Luchak J.M, L. et al. 2007)		*OK107-GAL4* (mushroom body)		12-19% (significant) increase
		MZ360-GAL4 (serotonergic neuronal)		1-12% (non-significant) increase
		Ddc-GAL4 (dopaminergic/serotonergic neuronal)		2-20% (non-significant) increase
		armadillo-GAL4 (low-level global)	1.5-2.0 fold (immunoblot)	13-29% (significant) increase
		C23-GAL4 (transverse flight muscles)		7-14% (significant) increase
(Orr, Radyuk et al. 2005)	GCLM	*Tub-GAL4* (high-level global)	60-100% (enzyme assay/immunoblot) 20-50% (GSH content)	24% mean lifespan increase
		D42-GAL4, elav-GAL, Appl-GAL4 (i.e. neuronal)		No impact on longevity

1.8.4 Selection Studies in *Drosophila melanogaster*

The link between reproductive timing and lifespan has been extensively studied. It is known that reproduction comes at a significant metabolic cost, especially in female flies where the physiological strain of egg production and laying is known to affect survival times. Early reproduction has been shown to be detrimental to both survival and fecundity (Prowse and Partridge 1997). This has been exploited in large-scale selection studies where populations of flies selected for late reproduction over a number of generations have given rise to specific long-lived strains (Arking 1987). These lines have been extensively studied and provide a good resource for studying genetic and environmental interventions and their effect on ageing. Arking's long- and short-lived fly lines are the most well characterised of these lines. Their longevity has been shown to vary according to the larval density of cultures (Dudas and Arking 1995) and other epigenetic issues (Arking, Dudas et al. 1993). The condition-dependent variation in longevity means that the data should be treated with caution (Baret, Le Bourg et al. 1996); analysis of subsequent work done over two decades with these lines should be examined in the context of culture conditions and genetic background. Nonetheless, these have proved a vital resource in the study of longevity in *Drosophila.*

Just as the overexpression of some components of the antioxidant defence system seem to have a developmental cost, longevity in these long-lived strains seems to come at the cost of developmental viability, with long-lived strains showing twice the developmental lethality

of normal-lived strains (Buck, Vettraino et al. 2000). This raises an important point with regards to lifespan studies: it is vital to look at extension in relation to any developmental cost there may be. If a putative gerontogene in *Drosophila* has extreme developmental effects, care must be taken in designing experiments that separate this from the effects on adult lifespan. It also raises a broader question about the relationship between cross-species interventions that extend lifespan – the cost of these to the organism and the species as a whole must be examined before drawing any conclusions as to the desirability of such an intervention.

These long- and short-lived lines are an invaluable resource for investigating the phenotypes associated with increased longevity. Arking's original long-lived lines also showed a significant level of resistance to paraquat (Arking, Buck et al. 1991). Although a causative relationship is not suggested, this trait is linked to the extended longevity phenotype as reverse selection removes both lifespan extension and paraquat resistance and Arking suggests that paraquat resistance is a useful biomarker of ageing (Arking, Buck et al. 1991). The underlying mechanisms behind this longevity extension are far from clear, however. In two lines with identical longevity and paraquat resistance phenotypes, both demonstrated transcriptional alterations in antioxidant gene expression, but different genes were transcriptionally active in each and there was post-translational up-regulation of at least one other antioxidant defence gene in one line (Arking, Burde et al. 2000). This implies that more than one molecular mechanism is responsible for elevated oxidative stress resistance (Arking, Burde et al. 2000). Nonetheless, extended longevity in at least one strain does correlate with enhanced levels antioxidant defence system components, including

accumulation of Cu/ZnSOD and reverse selection abrogates this (Arking, Burde et al. 2000), supporting the case for antioxidant involvement in lifespan determination. The relationship between paraquat resistance and longevity selection has been explored further by direct selection for paraquat resistance (Vettraino, Buck et al. 2001; Arking, Novoseltseva et al. 2002). The extended longevity pattern of these lines differed from those selected for late reproduction, with significantly lower developmental viability, suggesting that similar stress response mechanisms are generated by different molecular mechanisms (Vettraino, Buck et al. 2001; Arking, Novoseltseva et al. 2002). This is interesting as it implies that direct selection for upregulation of components of the antioxidant defence system (through paraquat exposure) leads to high levels of developmental lethality, supporting the hypothesis that there is an optimum balance point between oxidative damage protection and levels of ROS necessary to fulfil their roles in areas such as signalling within an organism.

It is important to look at these results in the context of whole organism physiology, however. Mockett *et al* studied the metabolic rate and potential of these lines and found that there was an increase in both, in addition to an increase in walking speed (Mockett, Orr et al. 2001). This means that the increased longevity phenotype is not purely a result of a compensatory slowing of metabolism. The lines also had different reactions to different stressors, suggesting that alleles underlying long and short life in these lines are linked to enhanced resistance to specific kinds of stress (Mockett, Orr et al. 2001). These models exemplify the complex relationship between stress resistance and longevity.

1.8.5 Other Genes that Affect Lifespan in *Drosophila melanogaster*

A wide range of genetic interventions significantly increase lifespan in *Drosophila melanogaster.* Those pertaining to the oxidative defence system have been discussed in detail above. Table 1.6 summarises genetic interventions specifically involved in the insulin signalling pathway which have been shown to increase longevity. Whilst these do provide large increases in lifespan, it should be noted that this is often accompanied by phenotypes such as sterility and dwarfism and so is not without organismal cost (Clancy, Gems et al. 2001; Tatar, Kopelman et al. 2001; Clancy, Gems et al. 2002). Table 1.6 summarises other genetic interventions which have had a positive effect on survival time in *Drosophila.* The large number of genes that affect lifespan reflects the polygenic nature of the phenomenon of ageing. This illustrates the importance of relating any increase seen in single gene manipulation experiments to the wider landscape of genetic interaction within the fruitfly before drawing any firm conclusions.

Table 1.5: Genetic interventions involved in downregulating the insulin signalling pathway that extend lifespan

Gene	Role & Intervention	Extension Reported	Reference
chico	Insulin receptor substrate (Null mutations)	Homozygous females, ↑48% mean lifespan Heterozygous males, ↑13% Homozygous males, reduced lifespan	(Clancy, Gems et al. 2001; Clancy, Gems et al. 2002)
Dilp	Signalling molecule (Ablation of producing cells)	↑35% Maximum lifespan	(Broughton, Piper et al. 2005)
InR	Insulin receptor (Hypomorphic)	↑85% in lifespan	(Tatar, Kopelman et al. 2001)
dFOXO	Forkhead transcription factor – insulin signalling pathway negatively regulates (Overexpression)	↑20-50% median lifespan ↑19% maximum lifespan	(Giannakou, Goss et al. 2004; Hwangbo, Gersham et al. 2004)
dSir2	Histone deacetylase – gene silencing in insulin signalling pathway (Overexpression)	↑57% median lifespan ↑25% in maximum lifespan	(Rogina and Helfand 2004)

Table 1.6: A review of genetic interventions that extend lifespan in *Drosophila*, excluding genes involved in the insulin signalling pathway

Gene	Role & Intervention	Extension Reported	Reference
Rpd3	Histone deacetylase (Loss of function)	↑50% median lifespan	(Rogina, Helfand et al. 2002)
puckered (puc)	JNK-specific phosphatise, downregulates JNK pathway (Loss of function)	↑ mean lifespan ↑ maximum lifespan	(Wang, Bohmann et al. 2005)
dPOSH	JNK signalling pathway (Overexpression)	↑14% mean lifespan	(Seong, Matsuo et al. 2001)
hep	JNK kinase, activates JNK (Overexpression)	↑50% mean lifespan ↑25% maximum lifespan	(Wang, Bohmann et al. 2003)
MsrA	Secondary oxidative defence enzyme, protein repair (Overexpression)	↑30% mean lifespan	(Ruan, Tang et al. 2002)
heat shock proteins (hsp)	Secondary oxidative defence, involved in removal of damaged proteins (Overexpression)	↑30% mean lifespan	(Morrow, Samson et al. 2004)
uncoupling proteins (UCP)	Pre-emptive oxidative defence, mitochondrial proteins that uncouple ETC (Overexpression)	↑28% median lifespan	(Fridell, Sanchez-Blanco et al. 2005)
Ecdysone receptor	Steroid hormone (Knockout)	↑40% median lifespan	(Simon, Shih et al. 2003)

1.8.6 Caloric Restriction

No system or pathway exists in isolation in an organism so it is important to consider the effects of any treatment or genetic manipulation in relation to any other systemic effects it may have. This is exemplified by studies carried out examining the effects of caloric restriction on glutathione levels in mammals. Mice raised under conditions of caloric restriction show elevated liver glutathione levels (48% higher than mice raised on a normal diet) (Taylor, Lipman et al. 1995). Mice show an age-related decrease in the ratio of reduced to oxidised glutathione (GSH:GSSG) in a variety of brain regions (Rebrin, Forster et al. 2007) and glutathione and glutathione-related enzyme activity declines with age in the rat kidney (Cho, Kim et al. 2003). Caloric restriction in both species leads to a reversal of these trends, with rats and mice showing resistance to these declines (Cho, Kim et al. 2003; Rebrin, Forster et al. 2007). This suggests that the redox imbalance that normally occurs during ageing is in some way ameliorated by caloric restriction. This is further supported by the fact that the GSH:GSSG ratio in the brain was lower in lines of mice that showed no lifespan increase than in age-matched extended longevity phenotype controls (Rebrin, Forster et al. 2007). This shows the difficulty in attributing a causative role to certain treatments and manipulations. It raises questions as to the mode by which caloric restriction extends lifespan. This is further complicated by the relationship between caloric restriction and the insulin signalling pathway.

1.8.7 Other Treatments that Affect Lifespan in *Drosophila melanogaster*

Table 1.7 below summarises the variety of treatments that have been applied to *Drosophila melanogaster* that have been found to have a positive effect on lifespan or stress resistance. The fact that lifespan can be affected by a variety of different external treatments highlights the importance of environmental factors when examining the phenomenon of ageing.

Table 1.7: A review of treatments that extend lifespan in *Drosophila melanogaster*

Paper Ref	Treatment	Delivery Method	Extension Reported	Effect on Stress Resistance
(Bahadorani, Bahadorani et al. 2008)	Vitamin A (retinol)	Dietary supplementation	None	Hyperoxia – reduced survival
(Bahadorani, Bahadorani et al. 2008)	Vitamin C (ascorbic acid)	Dietary supplementation	Significant extension at 20mM conc	Hyperoxia – reduced survival
(Bahadorani, Bahadorani et al. 2008)	Vitamin E (alpha Tocopherol)	Dietary supplementation	None	Hyperoxia – significant increase in resistance
(Zhao, Zhang et al. 2008)	Porphyrans	Dietary supplementation	Significant extension of between 6.1-8.68% in males and 8.93-12.29% in females	Heat stress – increased resistance
(Smith, Hoi et al. 2006)	Biotin deficiency	Diet deficient in biotin	30% increase in lifespan	Hydroxyurea and heat stress – increased resistance
(Kang, Benzer et al. 2002)	4-phenylbutyrate (PBA)	Dietary supplementation	Significant extension	Starvation and paraquat – increased resistance

1.8.8 Summary of Current Opinions on the Relationship between Stress Resistance and Lifespan in *Drosophila melanogaster*

The oxidative damage theory of ageing predicts that overexpression of components of the antioxidant defence system should lead to increases in both stress resistance and lifespan. The results discussed above show that this is not necessarily the case. It is now recognised that a complex situation exists where an organism may exist in a state of 'optimum balance' between protection from the harmful effects of ROS by antioxidant enzymes and the negative effects of these enzymes when expressed at high levels. How beneficial overexpression is appears to be dependent on the tissue where the enzyme is expressed, the level of overexpression and the levels of other antioxidant enzymes within that tissue. The most significant effects are seen in tissues where low levels of an enzyme are present. There are two possible explanations for these results. It is possible that the nervous system tissues are the 'rate-limiting' tissues in an organism and that accumulation of damage there is the defining factor in organismal ageing, with lifespan being limited by the absence of protective enzymes in these tissues and the accumulation of oxidative damage that this leads to. This would support the oxidative damage theory. It is also possible, however, that oxidative damage is not the limiting factor in ageing and lifespan. Overexpression of enzymes in tissues where they are not usually present could change the transcriptional profiles within these tissues in a more subtle way and this, in turn, could have a wider reaching effect at an organismal level, neither limited nor connected to damage prevention. It is certainly clear that no individual component of the antioxidant defence system should be examined in isolation without considering the results in the context of how the enzyme

affects the other antioxidant defence enzymes and that identical phenotypes may be underlain by multiple mechanisms.

This thesis aims to examine further the role of the oxidative defence system in lifespan, stress resistance and ageing by investigating the positive and negative implications of the alteration of glutathione titres in *Drosophila melanogaster* via overexpression of the subunits of GCL. It aims to dissociate this from developmental effects of these manipulations and examine the developmental consequences of the manipulation of one component of the antioxidant defence system.

2. METHODS AND MATERIALS

2.1. Fly Strains

Fly strains referred to in this thesis are listed in Table 2.1. In all cases, if an abbreviated form of the genotype has been used to refer to the strain in the text, it is listed in the table.

Table 2.1 Fly stocks used. All stocks were obtained from the Bloomington stock centre with the exception of the following: the UAS-GCL transgene strains were made in our laboratory by Dr. Pushpa Kansagra.; the Tub-GAL4 (Sb Tb) and Act(II)GAL4 (CyO GFP) driver strains were made according to the crossing schemes in Figure 3.2 and Figure 3.3; the *elav-GS* strain was a gift from T. Osterwalder (Yale University).

Fly Strain	Abbreviation	Comments
w^{1118};p{UAST GCLC T2.1.3 w^+}	UAS-GCLC	Containing Drosophila GCL transgenes in pUAST vector
w^{1118};p{UAST GCLM T7.3.1 w^+}	UAS-GCLM	
w^{1118};p{UAST GCLC T2.1.3 w^+}, p{UAST GCLM T7.3.1 w^+}	UAS-GCLC,GCLM	
w^-; ; P{D42-GAL4}	D42-GAL4	Expression in motor neurons
y^1 w^*; ; P{elav-GS}	elav-GS	Pan-neural expression induced by RU486 ingestion
w^{1118}	w^{1118}	Laboratory reference strain
y^1w^*;P{w^{+mC}=tubP-GAL4}LL7/ $TM3Sb^1$	Tub-GAL4	GAL4 driver with ubiquitous high level expression pattern
y^1w^*; P{w^{+mC}=Act5C-GAL4}17B F01/TM6B Tb	Act(III)GAL4	GAL4 driver with ubiquitous high level expression pattern
y^1w^*; P{w^{+mC}=Act5C-GAL4}17B F01/CyO	Act(II)GAL4	GAL4 driver with ubiquitous high level expression pattern
y^1w^*; P{w^{+mC}=TubP-GAL4}LL7/TM6C Sb^1 Tb	Tub-GAL4(Sb Tb)	GAL4 driver with ubiquitous high level expression pattern (see Figure 3.2)
y^1w^*; Act-GFP, CyO/ P{w^{+mC}=Act5C-GAL4}17B F01	Act(II)GAL4 (CyO GFP)	GAL4 driver with ubiquitous high level expression pattern (see Figure 3.3)
w^*; Sco/CyO, Act GFP		
$Eip74E^{DL1}$ st^1 p^p e^{11}/TM6C Sb^1 Tb^1		

2.2. Media

2.2.1 Standard Fly Media

150g Oatmeal, 100g molasses sugar, 24g agar, 16.8g Bakers' yeast and 2l dH_2O were brought to the boil in a bain-marie and simmered for 15 minutes. The media was then removed from the heat and, whilst stirring, 6.8g p-hydroxymethyl benzoic ester in 40ml ethanol was added. This media was then decanted into plastic fly bottles/vials which were subsequently bunged with cotton wool plugs and stored at 4°C. Unless otherwise stated, this was the standard media used for all background fly stock maintenance and all assays.

2.2.2 RU486 Fly Media

Standard fly media was made according to the protocol in Section 2.2.1 and allowed to cool slightly. RU486 was stored at -20°C as a 20mM Stock Solution in 80% ethanol (personal communications, T. Osterwalder). 25ml of the RU486 stock solution was pipetted into a falcon tube and bromophenol blue was dissolved in it. This enabled clear visualisation of the even mixing of the solution and the standard media. 25ml of the RU486 stock solution was then added to 1l of standard media to give a final concentration of 0.5mM. This media was then decanted into plastic fly bottles/vials which were subsequently bunged with cotton wool plugs and stored at 4°C for a maximum of 48 hours.

2.2.3 Embryo Collection Media

50g molasses sugar, 10g agar, 8.4g Bakers' yeast and 1l dH_2O were brought to the boil in a microwave and simmered for 5 minutes. The media was then removed, stirred and allowed to cool slightly. Then 3.4g p-hydroxymethyl benzoic ester in 20ml of ethanol was added. This media was decanted into petri dishes and stored at 4°C for use within 7 days.

2.3. Protocols

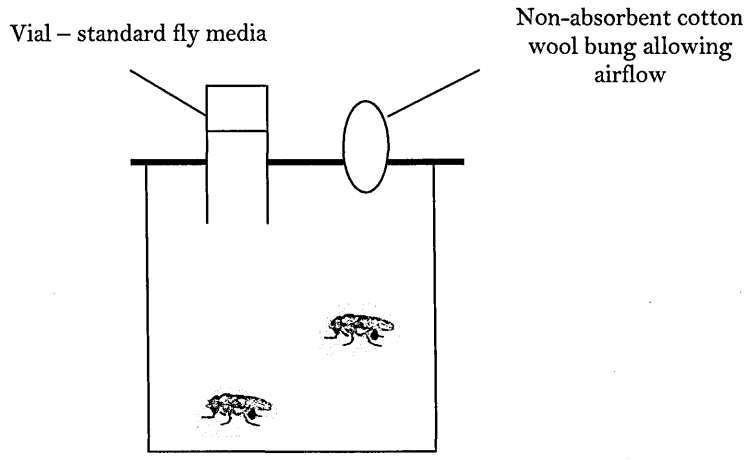
2.3.1 Standard Lifespan Protocol

Parental crosses were maintained on standard fly media (Section 2.2.1) at 25°C on a 12h light:12h dark cycle. The bottles contained 20 male flies and 20 female flies and the parents were cleared from the bottles after 4 days to ensure that larval density was low. The bottles were maintained at 25°C until eclosion. On the day of eclosion, male flies were collected using CO_2 anaesthesia in a temperature controlled laboratory at 25°C and transferred to 1000ml polypropylene tubs (Figure 2.1). The number of flies placed in each tub varied between approximately 40 and 120 dependent on the productivity of the parental bottles. The main consideration when setting up these tubs was to keep population size as consistent as possible between individual replicates in each experimental block. Separate parental bottles were used for the collection of female flies. On the day of eclosion, all freshly eclosed flies were transferred to fresh bottles by tipping without anaesthesia, where the females were aged for 4 days with males to allow for mating to occur. These flies were anaesthetised at day 4 and the females were transferred to tubs (Figure 2.1).

The lifespan tubs containing between 50 and 100 flies were maintained at 25°C on a 12h light:12h dark cycle. They were fed on standard fly media (Section 2.2.1) and vials were changed every second day. Dead flies were scored on a daily basis. All scoring and

maintenance of tubs was carried out inside a walk-in incubator during the 12h light period of the light:dark cycle.

Figure 2.1 Diagrammatic representation of tubs used for lifespan assays described in this thesis



2.3.2 Geneswitch Lifespan Protocol

Parental control crosses were maintained on standard fly media (Section 2.2.1) at 25°C on a 12h light: 12h dark cycle. The bottles contained 20 male flies and 20 female flies and the parents were cleared from the bottles after 4 days to control the larval density. The bottles were maintained at 25°C until eclosion. On the day of eclosion, male and female flies were transferred to fresh bottles of standard fly media by tipping without anaesthesia where they were aged together for 2 days. Prior lifespan experiments had followed a 4 day ageing protocol, however, it was determined that a 2 day ageing protocol was sufficient in this case and, combined with practical collection time constraints, this led to the decision to age for 2 days. They were then separated and collected using CO_2 anaesthesia and transferred to lifespan tubs (Figure 2.1). The lifespan tubs were maintained at 25°C on a 12h light:12h

dark cycle, fed on either standard media (Section 2.2.1) or RU486 media (Section 2.2.2) which was freshly made to avoid any chemical deterioration due to storage. Dead flies were scored on a daily basis. All scoring and maintenance was carried out inside a walk-in incubator during the 12h light period of the light:dark cycle.

2.3.3 Statistical Analysis of Lifespan Assays

All lifespan assays were analysed using the statistical package JMP Version: 4.0.2 (Academic). Kaplan-Meier survival curves were generated using the univariate survival analysis function and were subsequently analysed using a semi-parametric Log-Rank test, as is the convention in lifespan studies. The presence of censored data in some of the data sets influenced this choice, making the Log-Rank test a more powerful indicator of the significance of the differences between survival plots than a Wilcoxon test. Initially, each experimental dataset was analysed, grouped by genotype to determine whether the difference between the sets of Kaplan-Meier curves was significant. Once that had been determined, pairwise analysis was carried out within each experimental dataset that showed a significant difference, comparing the driven experimental lines with each individual control line used in the assay (pairwise comparison). In cases where more than one control line was included in the assay, the control lines were also compared with each other to determine the significance of any variations. In all cases, significance was defined as statistically significant when $p<=0.05$(*) and highly statistically significant when $p=<0.01$(**) or $p=<0.001$(***). Mean and median values were generated using JMP

Version: 4.0.2 (Academic) Summary Statistics. Maximum lifespan is defined as the day that the population reaches 90% mortality (Luchak J.M, L. et al. 2007). This is standard practice for lifespan analysis and gives a maximum figure that is representative of the population and not influenced by a small number of outlying long-lived individuals.

2.3.4 Chemical Stress Exposure Assay

Parental crosses were maintained on standard fly media (Section 2.2.1) at 25°C on a 12h light:12h dark cycle. The bottles contained 20 male flies and 20 female flies and the parents were cleared from the bottles after 4 days to prevent larval over-crowding. On the day of eclosion, male flies were collected using CO_2 anaesthesia and transferred to tubs as shown in Figure 2.2. These flies were maintained on standard fly media without chemical stressors at 25°C in an unlit incubator for 7 days post-eclosion to ensure that they had fully recovered from eclosion and that any immediate effects of anaesthesia had passed before exposing flies to additional stressors. During this period, the food media was replaced daily and flies were exposed to light once a day at the time of the vial change. From day 7, the regular media was removed and the hole was sealed. All dead flies were noted and censored from the assay. From day 7 onwards, flies were fed on the solutions listed below:

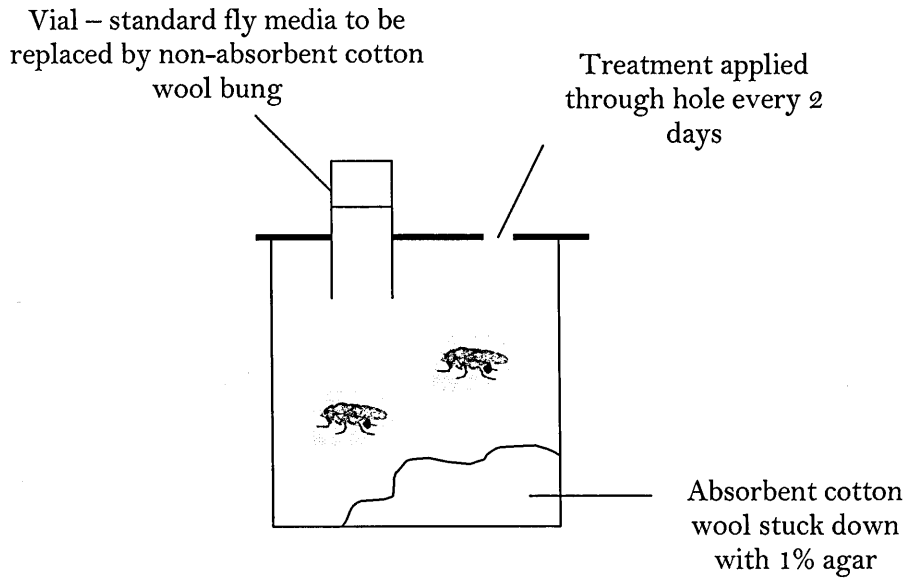
Table 2.2 Solutions used in chemical stress assays – concentrations listed are final concentration in dosing solution

	10% Sucrose	0.5mM RU486	5mM DEM	5mM Paraquat
Control 1	✔			
Control 2	✔	✔		
Control DEM	✔		✔	
Control Paraquat	✔			✔
DEM Experimental	✔	✔	✔	
Paraquat Experimental	✔	✔		✔

These solutions were freshly made up from stock solutions of DEM and paraquat to give the final concentrations shown in Table 2.2 every 2 days and applied to absorbent cotton wool by pipette via the dosing entry hole on every second day of the assay. Dead flies were scored daily.

DEM was stored at room temperature. Paraquat was stored at -20°C in ethanol as a 300mM stock solution. The stock solution bottle was wrapped in foil to minimise light exposure and the stress resistance assays were carried out in an unlit incubator as paraquat is light sensitive.

Figure 2.2 Diagrammatic representation of tubs used for chemical stress exposure assays



2.3.5 Determining the Concentration of Chemical Stressors

The 5mM concentration of paraquat used in the stress resistance assay described in Section 2.3.4 is the standard concentration used for oxidative stress resistance assays in *Drosophila* (Orr and Sohal 1993; Parkes, Elia et al. 1998; Mockett, Orr et al. 2001; Orr, Radyuk et al. 2005).

The 5mM concentration of DEM used in the stress resistance assay described in Section 2.3.4 was determined by conducting a 72 hour exposure gradient assay using w^{1118} flies (the laboratory reference strain). 5mM was chosen as the working concentration for all exposure assays as it was the highest concentration where flies showed no significant lethality at 72 hours exposure (data not shown). The rationale behind this choice was to

provide a low-level of oxidative stress over a period of time rather than a high lethal dose of the chemical.

2.3.6 Statistical Analysis of Survival Under Chemically Induced Stress

Statistical analysis of the survival curves generated by this assay was carried out in the same manner as the analysis of the lifespan curves in Section 2.3.3.

2.3.7 Parental Crosses for Embryonic and Larval Viability Assays

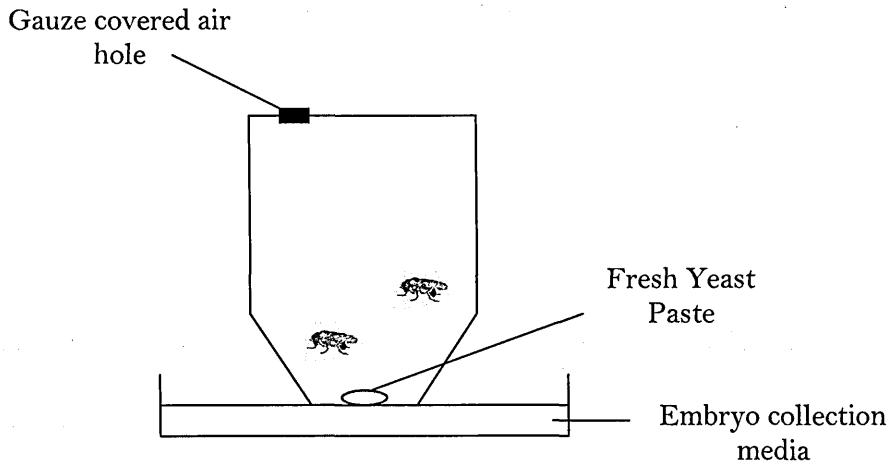
Virgin females and males from parental lines were collected and crossed in bottles on standard fly media (Section 2.2.1) and left to mate for 4 days at 25°C on a 12h light:12h dark cycle. The parents were then transferred to embryo collection population chambers for the embryo hatch assay (Section 2.3.8). These original bottles were kept and maintained until eclosion at 25°C on a 12h light:12h dark cycle for the absolute adult eclosion assay (Section 2.3.9)

2.3.8 Embryo Hatch Assay

Parental flies were maintained for the duration of the assay in population chambers as shown in Figure 2.3. Embryo collection media plates (Section 2.2.3) were used and the first and last plate of the day had fresh yeast paste applied to stimulate laying. Plates were

removed for scoring every 2 hours during the day. On removal, individual eggs were counted and their position was marked *in situ* on the plates. The plates were then transferred to 18°C and maintained at this temperature for the duration of the experiment. Plates were scored three times a day for hatched eggs (indicated by an empty chorion) for 48 hours.

Figure 2.3 Population chambers used for embryo harvesting



2.3.9 Adult Survival Assay

Absolute Survival

Parental bottles from the assay described in Section 2.3.7 above were maintained at 25°C on a 12h light: 12h dark cycle until eclosion. All adults were removed from these bottles and scored on a daily basis for 1 week post-first eclosion. At this point, the bottles were discarded to prevent F_2 progeny complicating the assay.

Seeded Vials

In order to follow a known number of larvae through to adulthood, plates containing fresh yeast paste were collected from the parental population bottles described in Section 2.3.7. These were maintained at 25°C until second and third instar larvae were visible. These larvae were harvested and placed in individual vials of standard fly media which were maintained at 25°C until eclosion. All adults that eclosed were collected using CO_2 anaesthesia and scored for genotype.

2.3.10 Assay to Identify Lethality Stage

Parental crosses were set up in bottles as described in Section 2.3.7. Embryos were harvested on plates of embryo collection media (Section 2.2.3) with fresh yeast paste applied to each plate.

1st Instar Hatching

Eggs were transplanted from the collection plate to a plate without yeast paste and placed at 18°C. These embryos were examined every 3 hours between 8 a.m. and 5 p.m. for a period of 48 hours and all hatched 1st instar larvae were collected and scored for genotype.

2nd and 3rd Instar Larval Survival

Plates were collected, wrapped in parafilm and aged at 25°C for 48h and 72h respectively for 2nd and 3rd instar larvae collection. Larvae were then floated out using a 3M saline

solution and separated into 2nd and 3rd instars on the basis of their anterior spiracles. These were then scored for genotype.

Pupal and Adult Survival

Parental flies were crossed in bottles on standard food media at 25°C. These flies were removed from the bottles after 4 days and the bottles were left to pupate at 25°C. All pupae were scored *in situ* for genotype and then the bottles were kept at 25°C until adults eclosed. All adults were collected and scored for genotype and sex.

Follow-Though Survival Assay

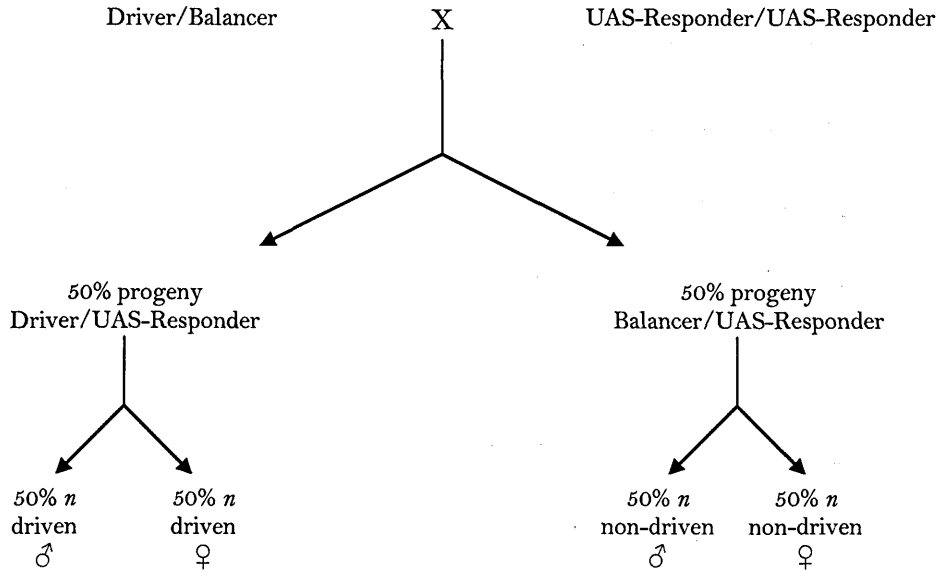
In order to follow a specific population of larvae through to adulthood, 2nd instar larvae were collected from parental crosses and separated according to genotype. These were subsequently transplanted to fresh vials as 3rd instar larvae and the survival percentage was noted. These larvae were kept at 25°C and the number of pupae for each group was scored *in situ*. All the successfully eclosed adults were scored for each genotype.

2.3.11 Statistical Analysis of Lethality Data

Comparisons of the mean survival values of the different genotypes was made using a 2-tailed two-sample t-test in Microsoft Excel (2003). The variances of the experimental and control lines were initially compared using an F-test two sample for variances in Microsoft

Excel (2003), depending on the result a t-test assuming either equal or unequal variance was applied.

Figure 2.4 Explanation of the relative percentages of progeny expected from crosses between a GAL4 driver strain carrying a balancer chromosome and a homozygous UAS-responder strain if the over-expression of the responder element had no effect on the survival of progeny.



Comparison between the expected survival ratio of the F_1 progeny of the crosses and the actual survival ratio and comparison between the expected sex distribution of the F_1 progeny and the actual distribution was made using a standard Chi-squared test in Microsoft Excel (2003). In all cases, the expected numbers were taken as 50% of the total number of progeny (*n*), as illustrated in Figure 2.4. Significance was defined as statistically significant when p<=0.05(*) and highly statistically significant when p=<0.01(**) or p=<0.001(***).

2.3.12 Removing Wolbachia Infection

In order to ensure that stocks cultures were free of the endosymbiotic bacteria *Wolbachia*, standard fly media was made according to the protocol in Section 2.2.1 and allowed to cool. This media was then supplemented with a tetracycline:ETOH solution giving a final concentration in the media of 0.003% (personal communication, David Clancy). This media was then cooled and the vials were bunged and stored at 4°C for use within 2 days. Flies were cultured on this media for a single generation. They were then returned to standard fly media without antibiotics and maintained on this media.

2.3.13 Suppliers and Catalogue Numbers

Reagent	Supplier	Catalogue Number
Agar	Sigma	A5054
Bromophenol blue	Sigma	B0126
Mifepristone (RU486)	Sigma	M8046
Parafilm	Sigma	P7793
Paraquat (methyl viologen)	Sigma	M2254
p-hydroxymethyl benzoic ester	Sigma	H6654
Sodium chloride	Sigma	S3014
Yeast extract	BD	288620

Equipment and Consumables	Supplier	Catalogue Number
Fly bottles	SLS	INC9002
Fly vials	Sarstedt Ltd	58-590
Non-absorbent cotton wool	Richardson's	C1690
Petri dishes (90mm)	Greiner	633185
Pipette tips 1-10 l	Starlabs	S111-3700
Pipette tips 1-200 l	Starlabs	S1110-1800
Pipette tips 100-1000 l	Starlabs	S1111-2721
Pipette tips 1-20 l	Starlabs	S1120-1810
Pipette tips 1-200 l	Starlabs	S1120-8810
Pipette tips 100-1000 l	Starlabs	S1126-7810
Polyethylene tubs and lids	Medfor Products Ltd	PN92
Sterile falcon tubes	Greiner	188271
Sterile pipette (single wrap), 5ml	Greiner	606180
Sterile pipette (single wrap), 10ml	Greiner	607180
Sterile pipette (single wrap), 25ml	Greiner	760180
1000ml transparent polypropylene jars with HDPE screw cap (lifespan tubs)	Medfor	PN92

3. CONSEQUENCES OF GLOBAL MANIPULATION OF GCL EXPRESSION IN *DROSOPHILA MELANOGASTER*

3.1. Introduction

The oxidative damage theory of ageing predicts that an enhancement of an organism's ability to deal with oxidative insult will positively influence longevity. Glutathione is often referred to as the body's master antioxidant and plays a key role an organism's oxidative defence system (Halliwell and Gutteridge 2007). Glutathione redox state is known to undergo age-related changes (reviewed in Section 1.5.2). Therefore, it can be hypothesised that an increase in an organism's ability to synthesise glutathione will result in a commensurate improvement in longevity. For this reason, it was decided to focus on the alteration of glutathione titres via manipulation of levels of the enzyme GCL, the rate limiting enzyme in the glutathione synthesis pathway in order to elucidate the role of this oxidative defence system in organismal lifespan.

Oxidative damage is an organism-wide problem. Even when the source of the generation of ROS is localised, for example to particular complexes in the mitochondrial ETC, the effects of these can be wide reaching (as discussed in Section 1.1). Consequently, the decision was initially made to use GAL4 drivers with high-level global expression patterns (detailed in Section 3.2). The rationale behind this approach was that global upregulation of enzyme activity leading to an increase in global glutathione levels should increase

organismal protection from oxidative damage, thereby reducing oxidative damage and increasing longevity (Orr and Sohal 1993; Parkes, Elia et al. 1998; Mockett, Orr et al. 1999; Mockett, Orr et al. 2001; Orr, Radyuk et al. 2005; Luchak, Prabhudesai et al. 2007).

The relationship between lifespan and sex is a complex one. In female flies, there is a trade-off between the benefits of reproduction and the metabolic cost to the individual of egg production and laying (Partridge, Green et al. 1987). This trade-off has implications for the longevity of female flies (Partridge, Green et al. 1987) and introduces further complicating factors to the lifespan assay such as whether females are virgin or mated or the rate/amount of egg production. For this reason, it was decided to focus the assay on male flies in the first instance.

GCL is a heterodimer of two sub-units: a catalytic subunit (GCLC) and a regulatory modifier subunit (GCLM) (as described in Section 1.5). This means there are a variety of implications regarding individual sub-unit over-expression that need to be taken into account when carrying out these kinds of manipulations. Although GCLM has no appreciable catalytic activity in isolation (Fraser, Saunders et al. 2002), it is possible that increasing the levels of GCLM above that of a wild-type fly could impact glutathione levels indirectly via an effect on the activity of endogenous GCLC. It has been proposed that GCLM is limiting for holoenzyme formation and that its over-expression is likely to enhance GCL activity (Krzywanski, Dickinson et al. 2004; Chen, Shertzer et al. 2005; Lee, Kang et al. 2006; Franklin, Backos et al. 2009). Therefore, the effects of over-expression of

the modifier subunit (GCLM) and the catalytic subunit (GCLC) individually were examined. *In vivo*, the holoenzyme complex formed by GCLC and GCLM is more efficient than the catalytic subunit alone (Fraser, Saunders et al. 2002). For this reason, the effects of co-over-expression of GCLC and GCLM in a recombinant fly line were examined to reduce the likelihood of lower endogenous levels of either GCLC or GCLM being the limiting factor in any longevity difference.

3.2. Fly Strains and Procedures

3.2.1 Fly Strains

The fly strains used in these experiments are detailed in Table 3.1 alongside the abbreviations that have been used in this chapter.

Table 3.1 Fly strains referred to in this chapter.

Fly Strain	Abbreviation	P-Element Chromosome	Description
w^{1118};p{UAST GCLC T2.1.3 w^+}	UAS-GCLC	II	Containing Drosophila GCL transgenes in pUAST vector
w^{1118};p{UAST GCLM T7.3.1 w^+}	UAS-GCLM	II	
w^{1118};p{UAST GCLC T2.1.3 w^+}, p{UAST GCLM T7.3.1 w^+}	UAS-GCLC,GCLM	II	
w^{1118}	w^{1118}		Laboratory reference strain
y^1w^*;P{w^{+mC}=tubP-GAL4}LL7/ TM3Sb^1	Tub-GAL4	III	Ubiquitous high level expression
y^1w^*; P{w^{+mC}=Act5C-GAL4}17B F01/TM6B Tb	Act(III)GAL4	III	High level ubiquitous expression
y^1w^*; P{w^{+mC}=Act5C-GAL4}17B F01/CyO	Act(II)GAL4	II	High level ubiquitous expression
y^1w^*; P{w^{+mC}=TubP-GAL4}LL7/TM6C Sb^1 Tb	Tub-GAL4(Sb Tb)	III	High level ubiquitous expression (see Figure 3.2)
y^1w^*; Act-GFP, CyO/ P{w^{+mC}=Act5C-GAL4}17B F01	Act(II)GAL4 (CyO GFP)	II	High level ubiquitous expression (see Figure 3.3)

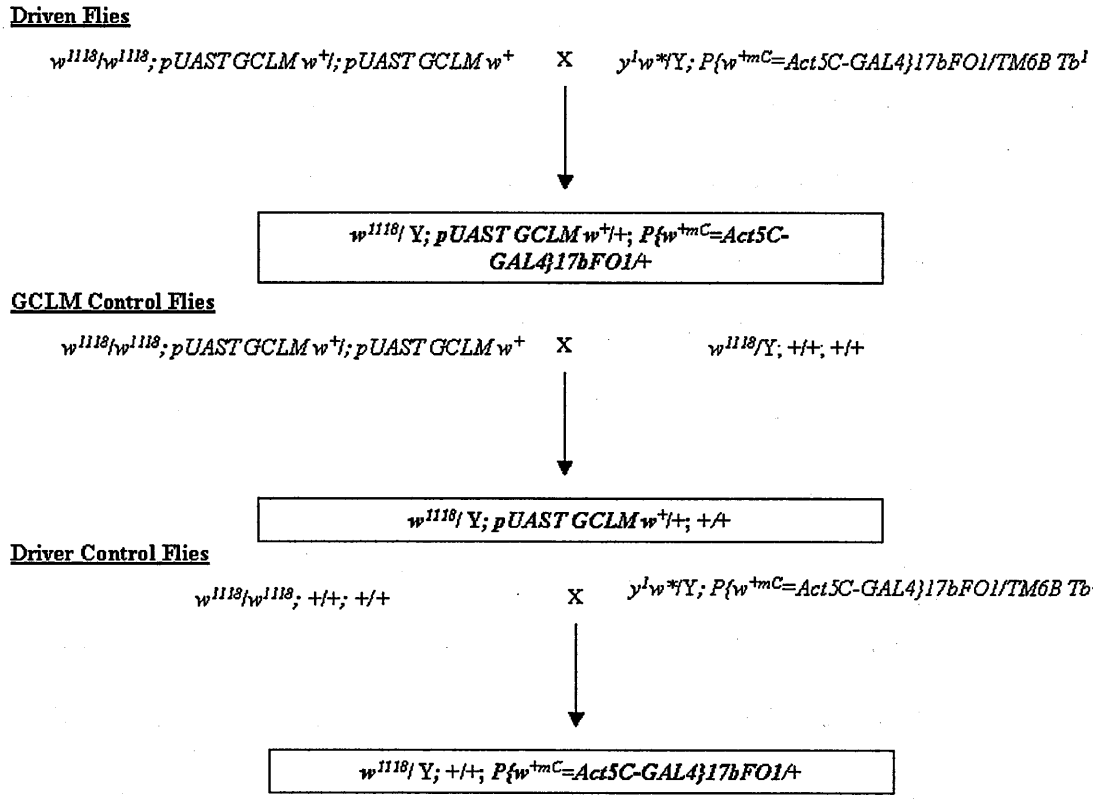
3.2.2 Procedures

Generation of Experimental Lines

In order to investigate whether global over-expression of GCLM altered longevity, flies were generated using the crossing scheme in Fig 3.1 below. The GAL4 drivers selected

(*Act(III)GAL4* and *Tub-GAL4*), with GAL4 fused to either an actin5C promoter or a tubulin promoter, led to ubiquitous high level expression at all stages of the lifecycle of the fly. This was previously verified by crossing with a *UAS-GFP* stock (Figure 3.6). It was decided to use both drivers for lifespan assays in order to assess whether any effects were specific to a certain driver rather than a result of ubiquitous over-expression of the modifier subunit itself. Male *UAS-GCLM/+; Act(III)GAL4/+* and *UAS-GCLM/+; Tub-GAL4/+* flies were collected, distinguishable from their siblings by the absence of dominant markers on the balancer chromosomes and darker eye pigmentation. Previous work by our laboratory using these drivers in combination with *UAS-GCLM* has demonstrated that it leads to an increase in levels of GCLM but no increase in total glutathione content (R.A. Akhtar, J. Fraser, unpublished results). This crossing scheme was replicated with the substitution of either *UAS-GCLC* or *UAS-GCLC,GCLM* in place of *UAS-GCLM* in order to assess the effects of all combinations of sub-unit over-expression. In all cases, over-expression is activated in a wild type background and is therefore expression above endogenous levels. Previous work by our laboratory driving *UAS-GCLC* with either of these drivers has demonstrated that these give a significant increase in levels of GCLC and in total glutathione content (Daniels, 2006) but that GCLM levels remain unaffected when GCLC is over-expressed in a wild type background. In addition, in flies where *UAS-GCLM* is over-expressed via crosses with these drivers, total glutathione levels remain unaffected (R.A. Akhtar, J. Fraser, unpublished results).

Figure 3.1 Crossing scheme used to generate flies for *Act(III)GAL4* driven *UAS-GCLM* lifespan experiments



In order to effectively distinguish different F_1 genotypes from the embryonic stage through all larval instars to adult flies, two driver strains were made carrying balancer chromosomes either constitutively expressing GFP (facilitating easy genotype identification from embryogenesis through all larval instars) or carrying two mutation markers which are easily distinguishable in 3rd instar larvae and in adults (Tb, which gives rise to larvae which are short and fat and Sb, which affects the bristles on adult flies making them short and stubbly) (Figure 3.2 and Figure 3.3).

Figure 3.2 Crossing scheme used to create strain containing *Act(II)GAL4* driver and GFP balancer chromosomes

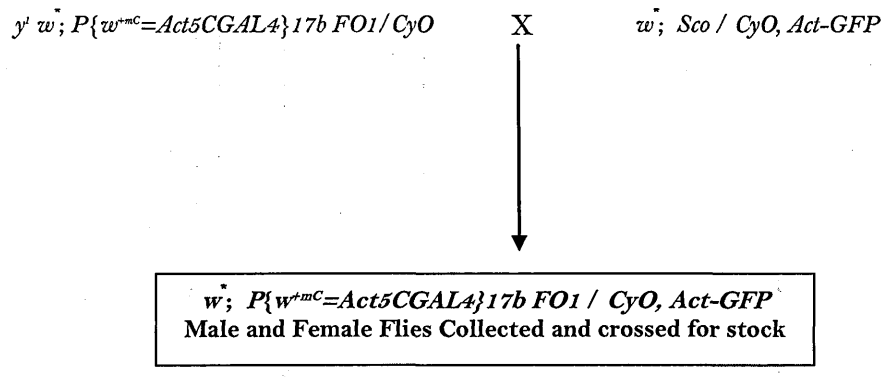
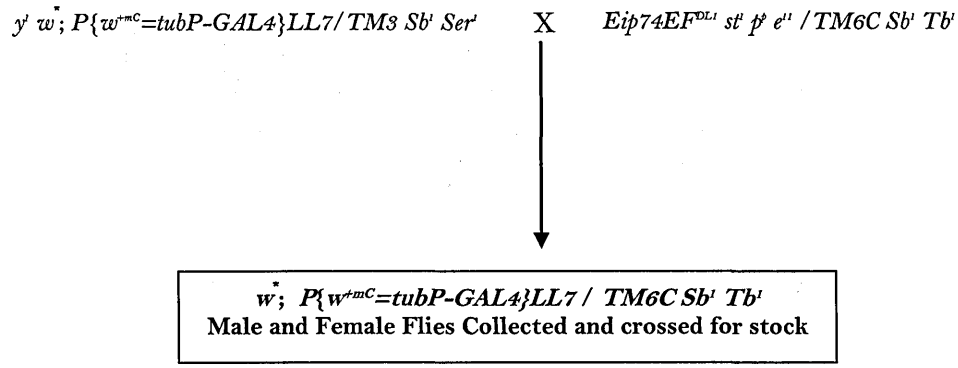


Figure 3.3 Crossing scheme used to create strain containing *Tub-GAL4* driver and balancer chromosome containing markers identifiable from 3rd larval instar to adult stages



Isogenisation of Fly Lines

The genetic background of fly lines can have a significant effect on longevity, independent of the gene of interest (see Section 1.7.5 Introduction). In order to minimise the background differences between the experimental line and the two control lines, all fly lines were isogenised by backcrossing to w^{1118}, our laboratory reference strain, for a minimum of 8 generations as illustrated in Figures 3.4 and 3.5 below. Exploiting recombination in female *Drosophila*, this strategy aimed to produce more genetically homogenous fly lines. After 8 generations of isogenisation crosses, the UAS responder lines should have minimal background genetic variation. The GAL4 driver lines are a more complicated issue – they

contain a balancer chromosome, used as a means of maintaining a viable stock of both strains where homozygosity for the GAL4 driver chromosome results in lethality. Therefore, a balancer chromosome needs to be re-introduced in the final cross. This means that, despite outcrossing both flies containing the driver chromosome and those containing the balancer to the w^{1118} laboratory reference strain, the isogenisation is imperfect due to suppression of recombination on the third chromosome of the TM3 and TM6B balancer crosses. The re-introduction of the balancer chromosome at the final cross has the potential to re-introduce genetic variation.

Figure 3.4 Crossing scheme used to isogenise UAS responder lines for lifespan assays

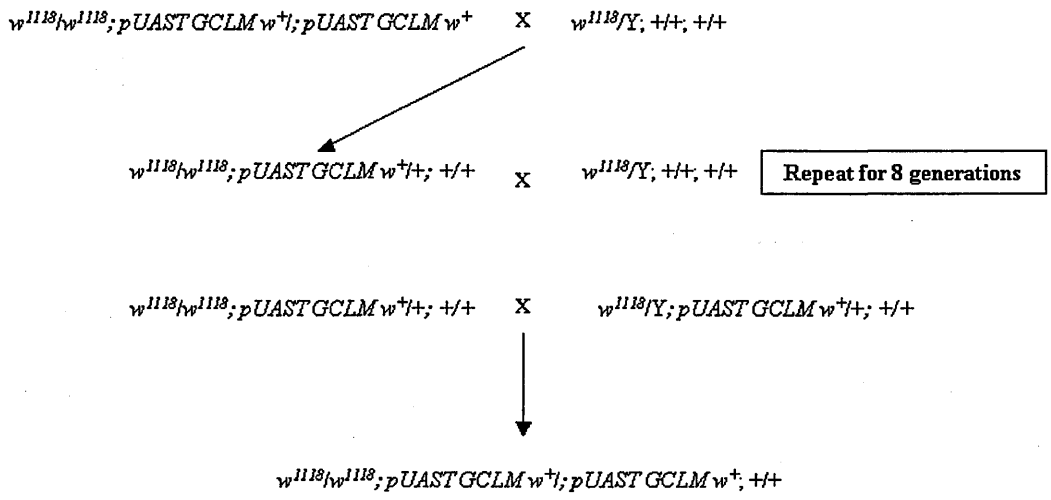
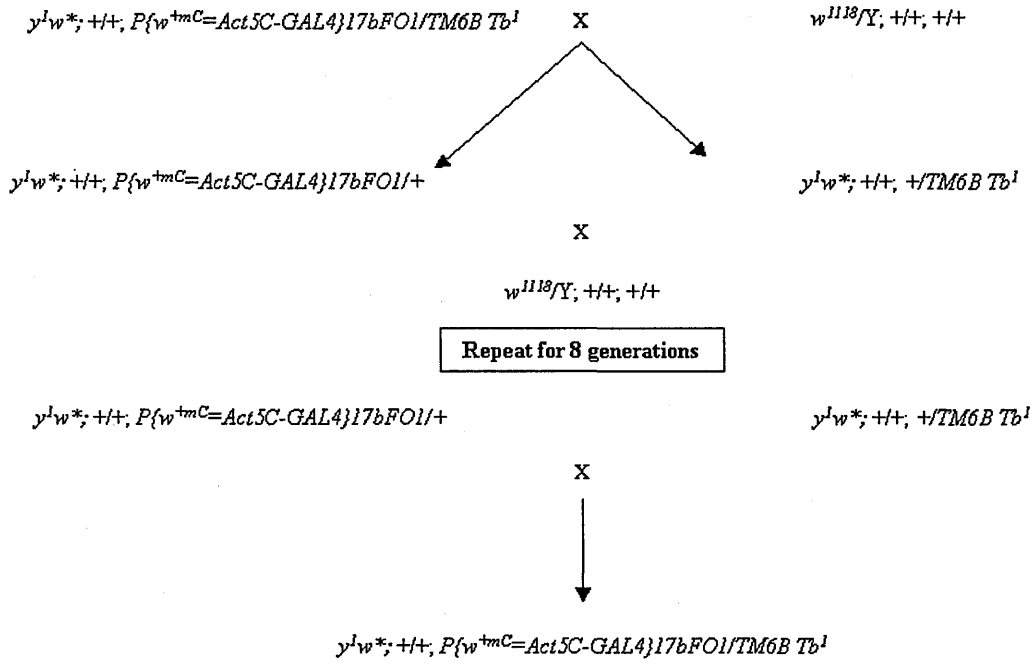


Figure 3.5 Crossing scheme used to isogenise GAL4 driver lines for lifespan assay



Checking Driver Expression

The expression pattern of the drivers was verified by crossing the driver lines to a UAS-GFP stock and imaging the progeny under a fluorescent microscope. The results are shown in Figure 3.6 and Figure 3.7 below.

Figure 3.6 Expression pattern of Actin(III)-GAL4. Panel (a) shows embryos from a cross between the Act(III)-GAL4 driver and a UAS-GFP line. GFP-positive embryos accounted for approximately 50% of all embryos scored. Panels (b)-(d) show larvae, pupae and adults of the genotype *P{w^{+mC}=UAS-GFP S65T}T2/+*; P{w^{+mC}=Act5C-GAL4}17B F01/+ (left hand side of the panel) and *P{w^{+mC}=UAS-GFP S65T}T2/+*; TM6B Tb/+ (right hand side of the panel).

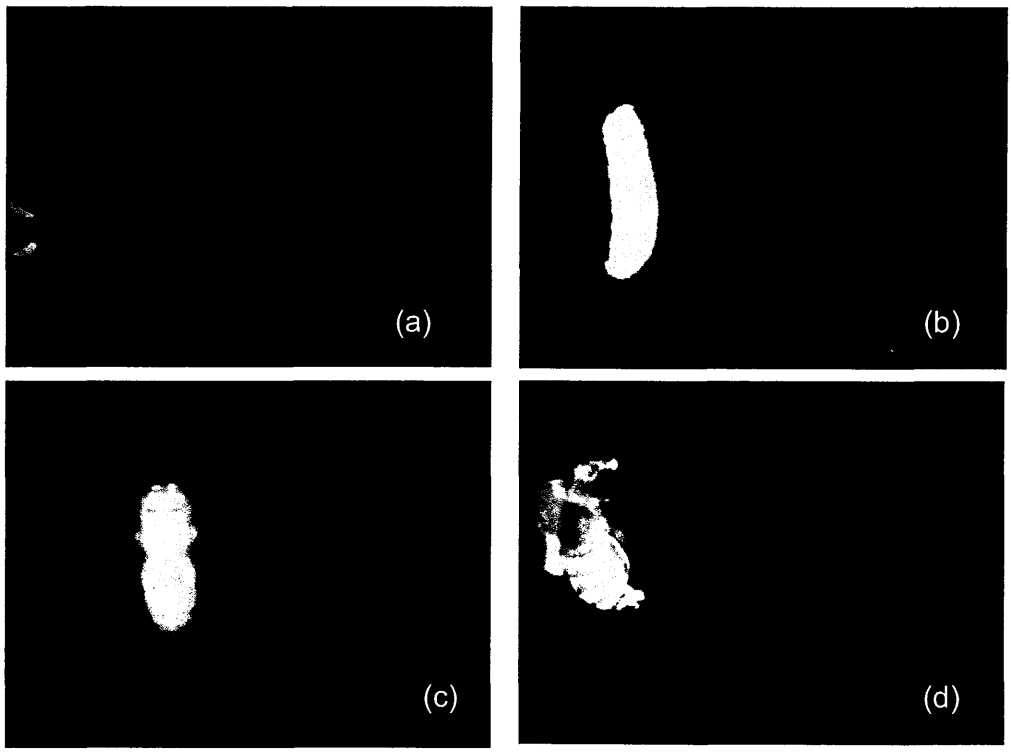
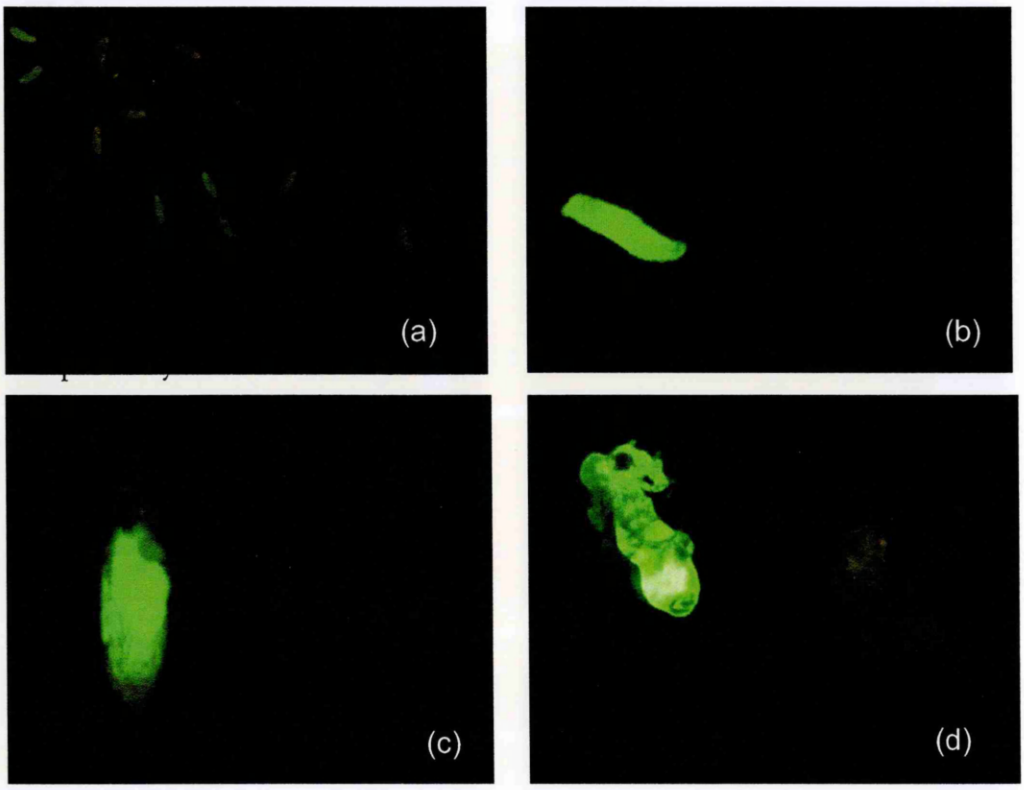


Figure 3.7 Expression pattern of Tubulin-GAL4. Panel (a) shows embryos from a cross between the Tubulin-GAL4 driver and a UAS-GFP line. GFP-positive embryos accounted for approximately 50% of all embryos scored. Panels (b)-(d) show larvae, pupae and adults of the genotype *P{w^{+mC}=UAS-GFP S65T}T2/+*; y^{1}w*; P{w^{+mC}=TubP-GAL4}LL7/+ (left hand side of the panel) and *P{w^{+mC}=UAS-GFP S65T}T2/+*; TM6C Sb^{1} Tb /+ (right hand side of the panel).



Lifespan assays were carried out according to the protocol described in Section 2.3.1 Methods and Materials. Statistical analysis of the data presented here was carried out according to the protocol described in Section 2.3.3 and 2.3.11 Methods and Materials.

Lethality Assays

Determination of the embryonic hatch rate, the lethal phase and the adult sex ratio were carried out as detailed in Sections 2.3.8, 2.3.9 and 2.3.10 (Methods and Materials). Statistical analyses were conducted as described in Section 2.3.11 (Methods and Materials).

Initial experimental results suggested confounding effects, possibly a result of a *Wolbachia* infection in the driver stock lines. It has been reported that *Wolbachia* infection is present in approximately 30% of stocks held at the Bloomington stock centre from which the driver lines used in these assays were acquired (Clark *et* al 2005). In order to assure that all driver strains were *Wolbachia*-free, flies from driver lines were first cultured for 2 generations on standard media containing 0.003% tetracycline (personal communication, David Clancy) (see Section 2.3.12, Methods and Materials). These strains were then cultured on standard media for at least two generations before the assay was carried out. In order to control for any effects the antibiotic culture may have had on experimental fly lines, all driver and responder lines used in this assay were passed through the same antibiotic treatment regime, regardless of whether *Wolbachia* infection was suspected.

3.3. Results

3.3.1 Ubiquitous Over-expression of GCLM

Over-expression of GCLM using ActinGAL4 driver

Initially, individual replicates of each genotype were analysed to provide a statistical analysis of the spread of replicate curves (see Fig 3.8 below). The data shown are from 3-4 replicates of approximately 50 flies per tub run simultaneously under identical conditions and fed on media from the same batch (Figure 3.8). In all cases, the number of replicates was variable as priority was given to maintaining as uniform a number of flies in each tub as possible rather than a consistent number of replicates. Where possible, 4 replicates were set up, however, if fewer flies eclosed from the parental bottles, fewer replicates were set up whilst maintaining consistent population density in each tub. There was no statistically significant difference between the lifespan of individual replicates of the driver control line. However, both the responder control line and the driven GCLM replicates were more widely spread and there was a statistically significant difference between the individual replicates (Table 3.2). Nonetheless, as a result of the uniformity of experimental conditions and timing, these data were treated as a single set for the purpose of this analysis. The long-term nature of lifespan experiments mean that a degree of spread between replicates is to be expected as a single increased mortality event early in the experiment in some replicates leads to an effect that persists throughout the lifespan of the experiment.

Figure 3.8 Individual replicates for Lifespan assay for *Act(III)GAL4* driver and *UAS-GCLM* responder line. Each replicate consists of an individual tub.

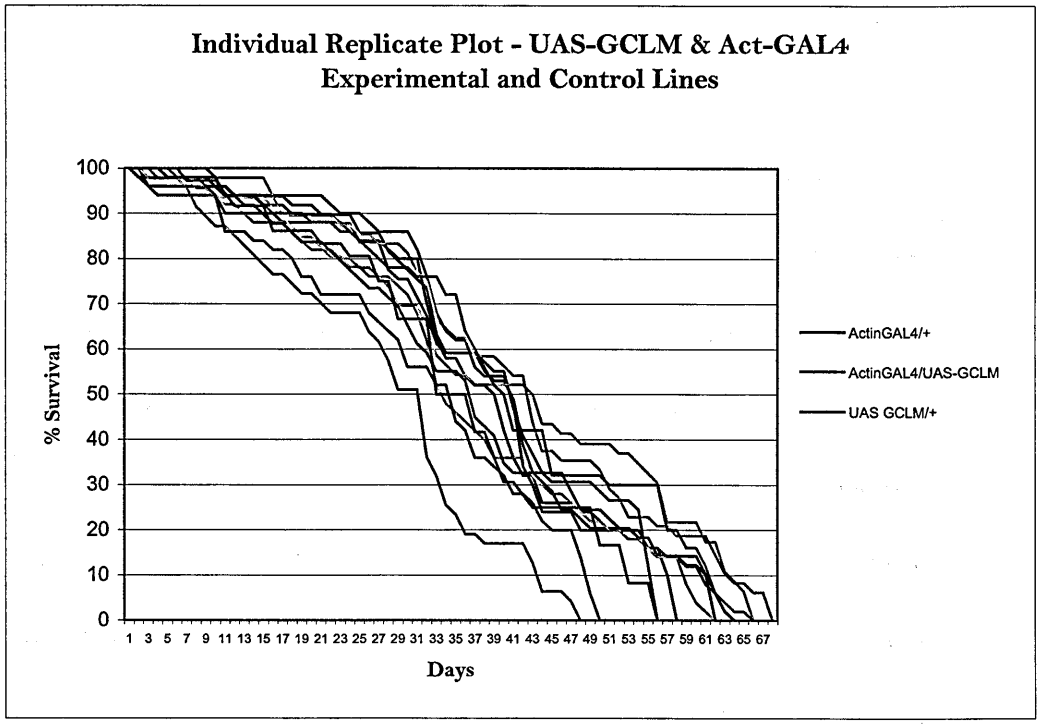


Table 3.2 Summary Statistics for individual replicates for lifespan for *Act(III)GAL4* driven *UAS-GCLM*

Genotype	Replicate	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value
Act(III)GAL4>UAS-GCLM	1	50	35.38	2.18266	33	58	<0.0001
	2	47	27	1.75987	31	53	
	3	46	40.11	2.6889	43	63	
	4	48	41.23	2.27191	42	62	
Act(III)GAL4/+	1	50	37.18	2.19864	38	60	0.8381
	2	49	38.04	2.11951	40	60	
	3	49	38.94	2.05068	40	60	
	4	50	38.18	1.84959	40	56	
UAS-GCLM/+	1	50	31.04	1.90316	34	48	<0.0001
	2	49	35.69	2.0994	36	54	
	3	50	40.26	2.2278	39	61	

When GCLM is over-expressed ubiquitously at high levels using an *Act(III)GAL4* driver, no significant improvement or impairment of lifespan is seen (Figure 3.9). Although driven flies show a 5.65% reduction in mean lifespan relative to driver control flies and a 0.76% increase in mean lifespan relative to the responder control flies, (the heterozygous actin control has a mean value of 38.08; the heterozygous GCLM control has a mean value of 35.66; *UAS-GCLM* driven by *Act(III)GAL4* has a mean value of 35.93) and there is a minor increase in maximum lifespan of 3.45% compared to driver control flies and 9.09% compared to responder control flies, the Kaplan Meier curves are not statistically significantly different (see Table 3.3 and Table 3.4, log rank test p values). Thus it appears that the over-expression of GCLM alone does not lead to any longevity increase. These results are not surprising, based on the knowledge that ubiquitous over-expression of GCLM did not lead to any increase in glutathione levels in flies (R.A. Akhtar, J. Fraser, unpublished results, discussed in Section 3.2.2).

Figure 3.9 Lifespan assay for *Act(III)GAL4* driver and *UAS-GCLM* responder lines and relevant controls. Each dataset is pooled data from 3-4 replicate tubs run simultaneously. There is no statistically significant difference between the curves (Log Rank Test, p=0.3648)

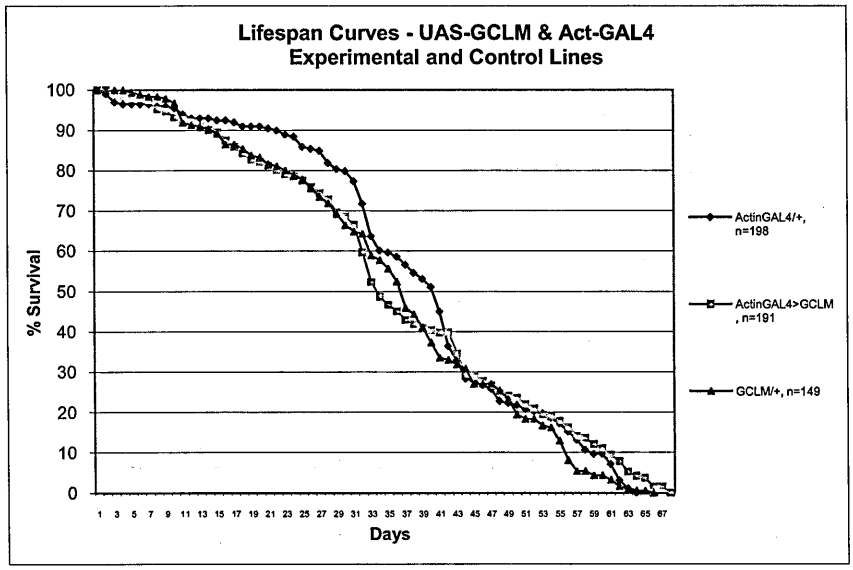


Table 3.3 Summary Statistics for Lifespan assay for *Act(III)GAL4* driver and *UAS-GCLM* experimental and control lines

Genotype	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value	DF
Act(III)GAL4/+	198	38.08	1.02222	40	58	0.3648	2
UAS-GCLM/+	149	35.66	1.233	36	55		
Act(III)GAL4>UAS-GCLM	191	35.93	1.18423	33	60		

Table 3.4 Pairwise comparison between genotypes for *Act(III)GAL4* driver and *UAS-GCLM* experimental and control lines showing percentage change in mean, median and maximum lifespan. In the case of both comparisons involving *Act(III)GAL4* driven *UAS-GCLM flies*, the figures refer to an increase or decrease in longevity of the driven flies relative to the control flies. In the case of the comparison of driver and responder control lines, the figures refer to an increase or decrease in longevity of the responder control flies relative to the driver control flies.

Comparison	% Change (Mean)	% Change (Median)	% Change (Max 90%)	Log Rank p-value	DF
Act(III)GAL4/+ vs Act(III)GAL4>UAS-GCLM	↓ 5.65	↓ 17.5	↑ 3.45	0.8390	1
UAS-GCLM/+ vs Act(III)GAL4>UAS-GCLM	↑ 0.76	↓ 8.33	↑ 9.09	0.2965	1
Act(III)GAL4/+ vs UAS-GCLM/+	↓ 6.36	↓ 10	↓ 5.17	0.1952	1

Over-expression of GCLM using TubulinGAL4 driver

In order to rule out any driver-specific effects, *UAS-GCLM* was crossed to a second GAL4 driver, *Tub-GAL4*, which also demonstrated ubiquitous high-level expression throughout all stages of the lifecycle of the fly. Once again, these data are pooled figures from 3-4 replicates of approximately 50 flies per tub run simultaneously under identical conditions and fed on media from the same batch (Figure 3.10). There was no statistically significant difference between the lifespan of each individual replicate for the control lines. However, there was a statistically significant difference between the individual replicates when the modifier subunit was ubiquitously over-expressed using the *Tub-GAL4* (Table 3.5). Nonetheless, as a result of the uniformity of experimental conditions and timing, these data were treated as a single set for the purpose of this analysis.

Figure 3.10 Individual replicates for Lifespan assay for *Tub-GAL4* driver and *UAS-GCLM* responder. Each replicate consists of an individual tub.

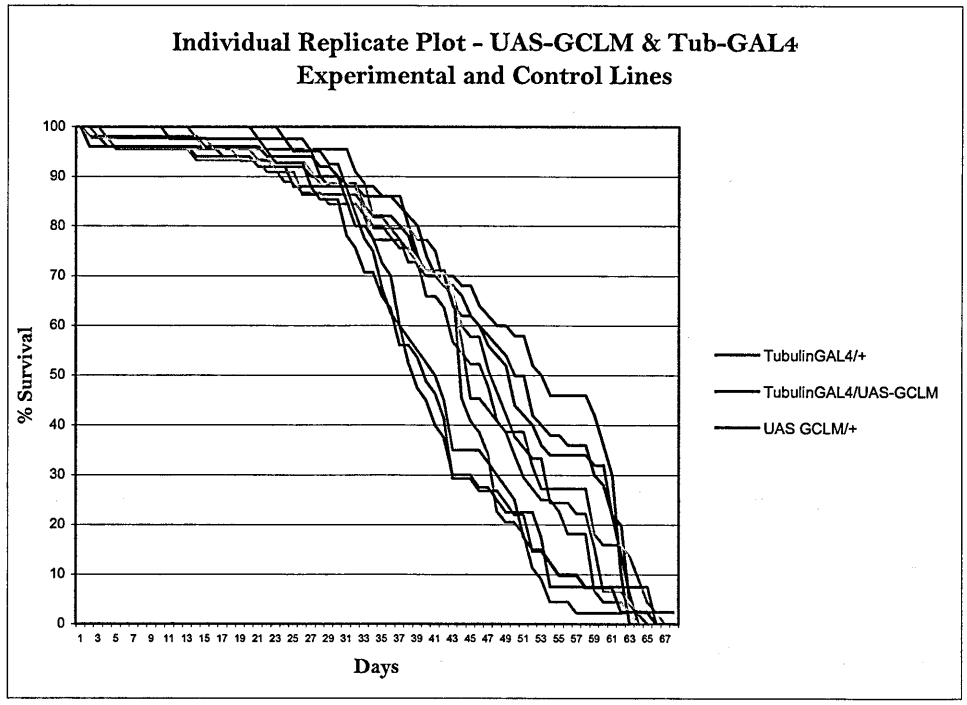


Table 3.5 Summary Statistics for individual replicates for lifespan for *Tub-GAL4* driven *UAS-GCLM*

Genotype	Replicate	N (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value
Tub-GAL4>UAS-GCLM	1	44	40.98	1.84451	43	52	0.0386
	2	45	44.04	2.20513	47	59	
	3	44	46.05	1.89217	44	63	
	4	44	43.56	1.94927	46	58	
Tub-GAL4/+	1	50	46.46	2.21207	49	62	0.9643
	2	50	46.6	2.12987	49	62	
	3	50	48.72	2.02858	52	61	
UAS-GCLM/+	1	40	41.23	1.64842	40	54	0.8344
	2	44	41.34	2.36204	40	54	
	3	40	40.63	1.69149	38	54	

When *UAS-GCLM* is driven using a *Tub-GAL4* driver (Figure 3.11), the situation is more complicated than the results obtained from the *Act(III)GAL4* crosses above. In this case, the Kaplan-Meier curves show a highly statistically significant difference (Table 3.6). This is reflected by differences in mean values (the heterozygous Tub-GAL4 driver control has a mean value of 47.26 days; the heterozygous GLCM control has a mean value of 41.07 days; the *Tub-GAL4* driven *UAS-GCLM* line has a mean value of 43.66 days), median values (51 days, 39 days and 44 days respectively for heterozygous driver, heterozygous responder and *Tub-GAL4* driven *UAS-GCLM*) and maximum values (62 days, 54 days and 59 days respectively for heterozygous driver, heterozygous responder and *Tub-GAL4* driven *UAS-GCLM*) (Table 3.6).

Pairwise comparison of all genotypes shows that there is a highly statistically significant difference between the heterozygous driver control and the *Tub-GAL4* driven *UAS-GCLM* Kaplan-Meier curves (Table 3.7 log rank p-values). However, it is the *Tub-GAL4* driven line that is impaired relative to the heterozygous driver control line with a 7.62% reduction in mean values, a 13.73% reduction in median values and a 4.84% reduction in maximum values. Despite increases of 6.31% in mean lifespan, 12.82% in median lifespan and 9.26% in maximum lifespan relative to the heterozygous responder control, the difference in the Kaplan-Meier curves is not significant (Table 3.7 log rank p-values). This indicates that the significant difference in lifespan seen between the driver control and the driven flies is unlikely to be linked to the experimental over-expression of GCLM but is more likely to be a result of some component of the genetic background of the lines involved. The fact that the *Tub-GAL4* driver heterozygous control line demonstrates the greatest

longevity, the *UAS-GCLM* heterozygous control line the shortest, and that the *Tub-GAL4* driven *UAS-GCLM* line lies between the two implies that the defining factor in the longevity of these lines is the presence or absence of the *Tub-GAL4* chromosome rather than the level of GCLM expression. Any minor advantage that GCLM over-expression gives these flies may be masked by the highly significant increase in lifespan associated with the presence of the *Tub-GAL4* chromosome. This certainly raises the question of whether it is ever possible to truly isogenise distinct strains by backcrossing and indicates that effective study of longevity in *Drosophila* requires a driver system that removes the confounding factor of potential differences in the genetic background of the strains used, such as the Geneswitch system used in the results presented in Chapter 5 of this thesis.

Figure 3.11 Lifespan assay for *Tub-GAL4* driver and *UAS-GCLM* responder lines and relevant controls. Each dataset is pooled data of 3 or 4 individual tubs run simultaneously. The curves show a statistically significant difference (Log Rank Test, p=<0.0001). Pairwise comparison of curves is detailed in Table 3.6.

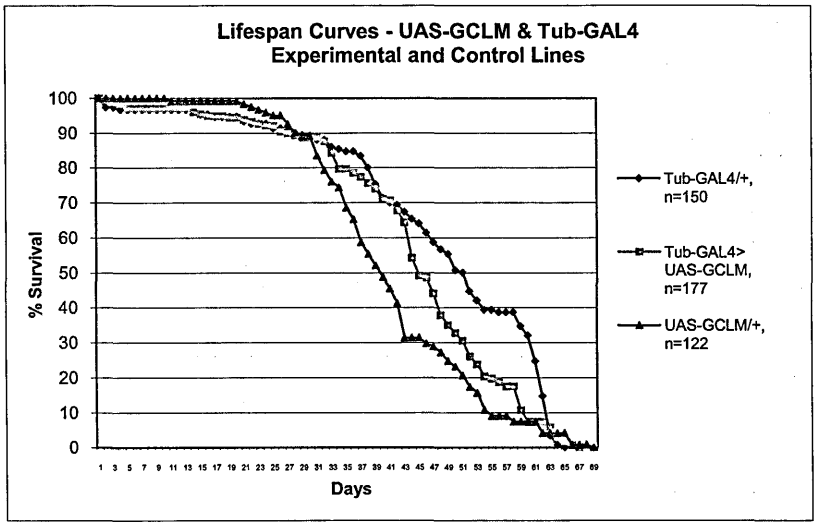


Table 3.6 Summary Statistics for Lifespan assay for *Tub-GAL4* driver and *UAS-GCLM* experimental and control lines

Genotype	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value	DF
Tub-GAL4/+	150	47.26	1.22145	51	62	<0.0001	2
UAS-GCLM/+	124	41.07	1.12404	39	54		
Tub-GAL4>UAS-GCLM	177	43.66	0.99113	44	59		

Table 3.7 Pairwise comparison between genotypes for lifespan for *Tub-GAL4* driver and *UAS-GCLM* experimental and control lines. In the case of both comparisons involving *Tub-GAL4* driven *UAS-GCLM* flies, the figures refer to an increase or decrease in longevity of the driven flies relative to the control flies. In the case of the comparison of driver and responder control lines, the figures refer to an increase or decrease in longevity of the responder control flies relative to the driver control flies

Comparison	% Change (Mean)	% Change (Median)	% Change (Max 90%)	Log Rank p-value	DF
Tub -GAL4/+ vs Tub -GAL4>UAS-GCLM	↓ 7.62	↓ 13.73	↓ 4.84	0.0021	1
UAS-GCLM/+ vs Tub -GAL4>UAS-GCLM	↑ 6.31	↑ 12.82	↑ 9.26	0.0503	1
Tub -GAL4/+ vs UAS-GCLM/+	↓ 13.10	↓ 23.53	↓ 12.90	<0.0001	1

3.3.2 Ubiquitous Over-expression of GCLC

Over-expression of GCLC using Act(III)GAL4 driver

Initially, individual replicates were assessed and a decision was made regarding pooling and censoring of data. Uniformly across all lines there was a small but sharp initial death event in the first 5 days of the assay. It is highly unlikely that this is a result of the experimental manipulation of GCLC expression as this early mortality was common to both experimental and control lines. It is a possibility that it was an adverse response to the CO_2 anaesthesia and collection routine, an effect often seen in lifespan assays. As differing responses to initial anaesthesia influence lifespan curve differences for the remainder of the experiment, the decision was made to censor the first 4 days of data to remove the effect of this early mortality event from the analysis. The data shown in the graphs and tables below are post-censor. These data are pooled figures from 3-4 replicates of approximately 50 flies per tub and, as in previous experiments, these were run simultaneously under identical conditions and fed on media from the same batch (Figure 3.12). There was no significant difference between replicates of the *ActinGAL4* control line or the *UAS-GCLC* control line. However, as shown in Table 3.8, there was a significant difference between replicates for the *Act(III)GAL4* driven *UAS-GCLC* experimental lines. Nonetheless, as a result of the uniformity of experimental conditions and timing, these data were treated as a single set for the purpose of this analysis.

Figure 3.12 Individual replicates for Lifespan assay for *Act(III)GAL4* driver and *UAS-GCLC* responder lines. Data were censored for deaths occurring prior to day 5. Each replicate consists of an individual tub.

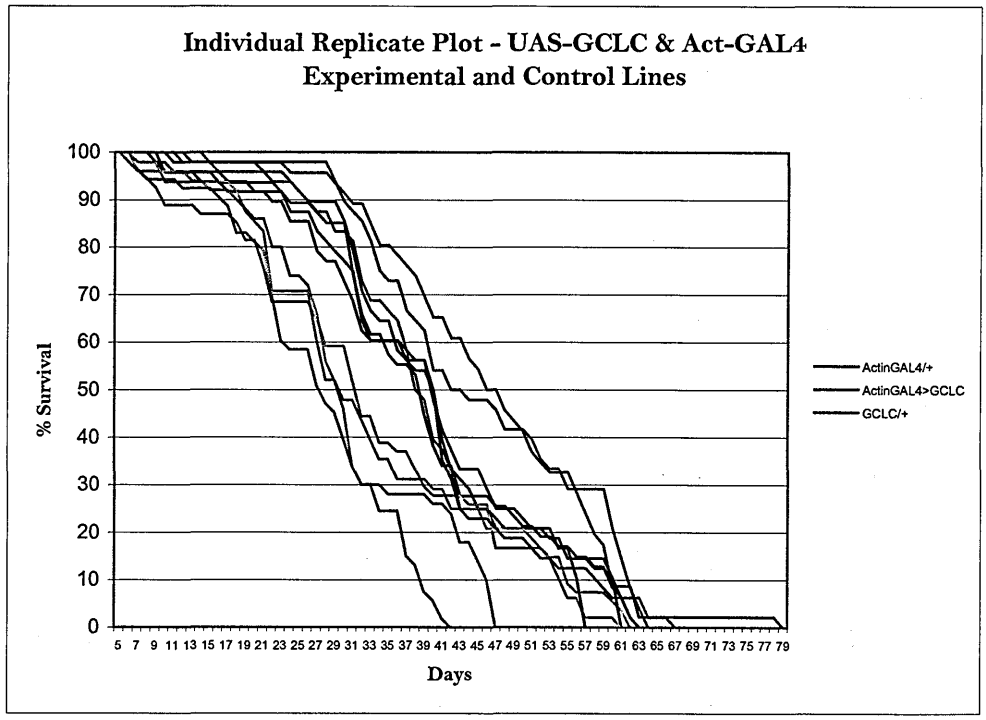


Table 3.8 Summary Statistics for lifespan for individual replicates for *Act(III)GAL4* driven *UAS-GCLC*

Genotype	Replicate	n (Flies)	Cen	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value
Act(III)GAL4>UAS-GCLC	1	53	2	27.49	1.24228	28	39	0.0026
	2	50	2	30.88	1.49929	30	46	
	3	48	2	33.29	2.01126	30	54	
	4	55	1	32.67	2.11696	32	55	
Act(III)GAL4/+	1	47	3	39.40	1.91968	39	60	0.8668
	2	48	1	38.81	2.0156	41	61	
	3	48	1	39.71	1.94083	40	61	
	4	48	2	39.71	1.57241	40	56	
UAS-GCLC/+	1	48	2	39.83	1.69906	39	58	0.0554
	2	48	0	45.75	1.86668	44	62	
	3	46	0	46.78	1.84886	48	60	

When GCLC is over-expressed ubiquitously at high levels using an *Act(III)GAL4* driver, contrary to the expectations discussed in Section 3.1, it leads to a significant impairment of longevity reflected in reduced mean values (the heterozygous actin control has a mean value of 39.41; the heterozygous *UAS-GCLC* control has a mean value of 44.08; the *Act(III)GAL4* driven *UAS-GCLC* experimental line has a mean value of 31.05), median values (40 days, 42 days and 30 days respectively for the *Act(III)GAL4* driver control, the UAS-responder control and the driven flies) and maximum values (58 days, 61 days and 47 days respectively for the *Act(III)GAL4* driver control, the UAS-responder control and the driven flies) as illustrated in Figure 3.13 and Table 3.9.

Pairwise comparison of all genotypes shows that there is a highly statistically significant difference in the Kaplan-Meier curves when the driven flies are compared to the heterozygous driver control (a 21.21% reduction in mean lifespan, a 25% reduction in median lifespan and an 18.97% reduction in maximum lifespan) and the heterozygous responder control (a 29.56% reduction in mean lifespan, a 28.57% reduction in median lifespan and a 22.98% reduction in maximum lifespan) (Table 3.10). The control lines are also highly statistically significantly different from each other although the longevity of the driven flies is impaired relative to either control.

Figure 3.13 Lifespan assay for *Act(III)GAL4* driver and *UAS-GCLC* responder lines and relevant controls. Data were censored for deaths occurring prior to day 5. Each dataset is pooled data from 3-4 replicate tubs run simultaneously. The survival plots are significantly different (Log Rank test, p=<0.0001). Pairwise comparison is detailed in Table 3.9.

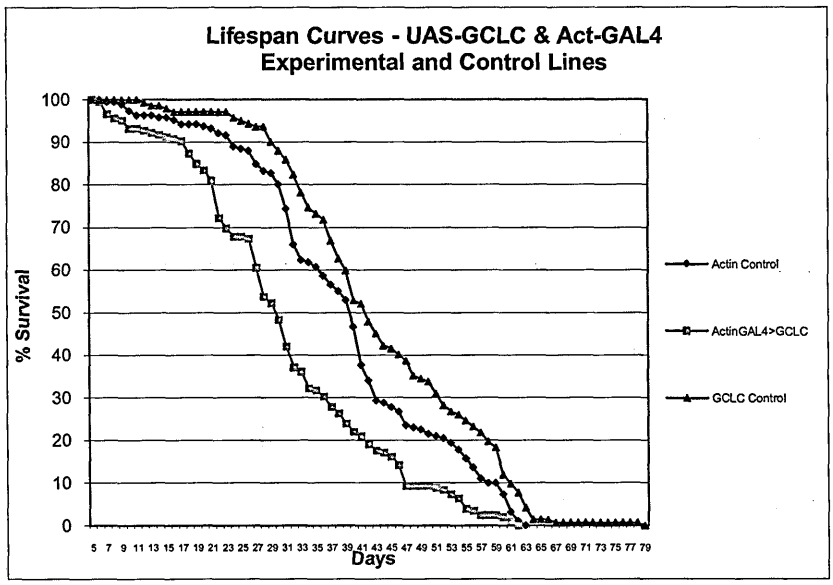


Table 3.9 Summary Statistics for Lifespan assay for *Act(III)GAL4* driver and *UAS-GCLC* experimental and control lines

Genotype	n (Flies)	Cen (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value	DF
Act(III)GAL4/+	191	7	39.41	0.92773	40	58	<0.0001	2
UAS-GCLC/+	142	2	44.08	1.06677	42	61		
Act -GAL4>UAS-GCLC	206	7	31.05	0.8874	30	47		

Table 3.10 Pairwise comparison between genotypes for lifespan for *Act(III)GAL4* driver and *UAS-GCLC* experimental and control lines. In the case of both comparisons involving *Act(III)GAL4* driven *UAS-GCLC* flies, the figures refer to an increase or decrease in longevity of the driven flies relative to the control flies. In the case of the comparison of driver and responder control lines, the figures refer to an increase or decrease in longevity of the responder control flies relative to the driver control flies.

Comparison	% Change (Mean)	% Change (Median)	% Change (Max 90%)	Log Rank p-value	DF
Act -GAL4/+ vs Act -GAL4>UAS-GCLC	↓ 21.21	↓ 25	↓ 18.97	<0.0001	1
UAS-GCLM/+ vs Act -GAL4>UAS-GCLC	↓ 29.56	↓ 28.57	↓ 22.98	<0.0001	1
Act -GAL4/+ vs UAS-GCLC/+	↑ 11.85	↑ 5	↑ 5.17	0.0010	1

However, the situation is more complex than simply a reduction in overall longevity. Mortality curves have three distinct phases that are responsible for the distinctive shape of the curve: an initial plateau stage, a steep incline and a late shallower incline. In the initial platform phase of the curve, mortality is constant rather than accelerating. As the incline changes and becomes steeper, this indicates that mortality accelerates – the steeper the curve, the greater the rate of acceleration. The point of turn between the plateau and the steep curve marks what is often referred to as the onset of 'ageing'. This profile reflects the fact that the chances of death increase with age. This is explained in more detail in Section 2.3.5 Methods and Materials.

Act(III)GAL4 driven *UAS-GCLC* shows no initial plateau phase, although the slope of the curve is not as pronounced as the steepest portion, and therefore high initial mortality compared to control lines. Death in this portion of the curve where mortality is typically constant is age-independent mortality and therefore a result of something that happens during development or very soon after eclosion which impairs early survival. It is possible that this effect is more extreme than is evidenced in Figure 3.12 and that censoring of the initial 4 days' data masks a more extreme effect. Nonetheless, as there is no way of separating this mortality from that experienced in both control lines, censoring the initial 4 days of the experiment is still justifiable but must be borne in mind when interpreting these data. The turning point indicating the onset of age-dependent mortality (or 'ageing') is also much earlier for the driven *UAS-GCLC* line (at approximately day 21 in comparison to approximately day 30-32 for control lines) and the slope of the curve is steeper, indicating a faster rate of ageing. There is a late flattening of the survival curve for *Act(III)GAL4* driven

UAS-GCLC from approximately day 51 which is not seen in either control line, which could indicate that flies which manage to overcome the causes of increased initial mortality and a faster rate of ageing may have some later-life advantage that means mortality is decreased relative to control lines, although this is far from certain as the maximum lifespan is markedly shorter (a reduction of 18.97% relative to the driver control and 22.98 relative to the responder control) for these flies.

Over-expression of GCLC using Tub-GAL4 driver

Once again, to rule out driver-specific impairment effects, *UAS-GCLC* was crossed to a second high-level ubiquitous driver, Tub-GAL4. Initially, individual replicates were assessed for degree of spread (Figure 3.14). As there was no initial mortality in this experiment, the immediate post-eclosion period was not censored. The independent replicates were pooled for the purpose of this assay as, although there was a statistically significant difference between *UAS-GCLC* control replicates (Table 3.11), experimental conditions and timing were uniform, these data were treated as a single set for the purpose of this analysis.

Figure 3.14 Individual replicates for lifespan assay for Tub-GAL4 driver and UAS-GCLC responder lines. Each replicate consists of an individual tub.

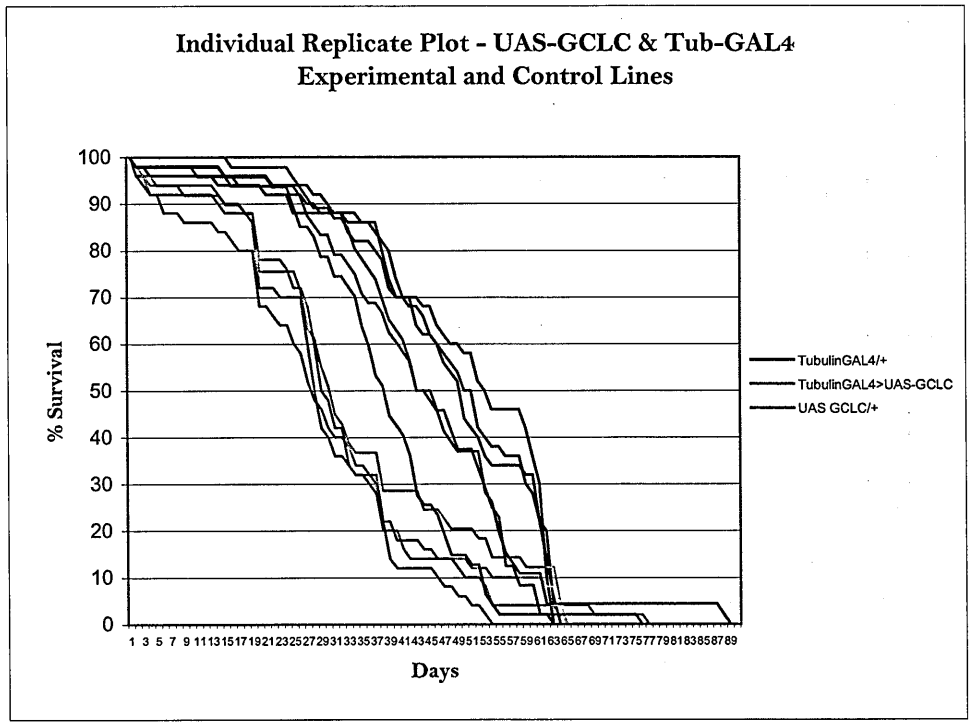


Table 3.11 Summary Statistics for lifespan for individual replicates for *Tub-GAL4* driven *UAS-GCLC*

Genotype	Replicate	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value
Tub-GAL4>UAS-GCLC	1	50	27	1.92471	27	45	0.2357
	2	50	30.06	2.10469	27	49	
	3	50	30.78	2.15033	29	53	
	4	49	33.10	2.41404	30	63	
Tub-GAL4/+	1	50	46.46	2.21207	49	62	0.9643
	2	50	46.6	2.12987	49	62	
	3	50	48.72	2.02858	52	61	
UAS-GCLC/+	1	47	37.26	1.63322	38	52	0.0021
	2	46	44.93	2.09909	44	61	
	3	48	42.40	2.06386	45	57	
	4	45	44.53	1.80644	45	58	

The highly statistically significant longevity impairment seen when the catalytic subunit is over-expressed ubiquitously at high levels using an *Act(III)GAL4* driver is replicated when *UAS-GCLC* is over-expressed using a Tub-GAL4 driver with reduction in mean values (the heterozygous *UAS-GCLC* control has a mean value of 42.24; the heterozygous tubulin control has a mean value of 47.26; the *Tub-GAL4* driven *UAS-GCLC* experimental line has a mean value of 30.22) median values (51 days, 42 days and 28 days respectively for driver control flies, responder control flies and driven flies) and maximum values (62 days, 57 days and 53 days respectively for driver control flies, responder control flies and *Tub-GAL4* driven *UAS-GCLM* (Figure 3.15 and Table 3.12).

Pairwise comparison of all genotypes shows that there is a highly statistically significant difference between the *Tub-GAL4* driven *UAS-GCLC* line and both controls and that the control lines are significantly different from each other (Table 3.13) The line with the greatest longevity is again the heterozygous driver control line, as was the case when it was used to drive *UAS-GCLM* (see Section 3.3.1 above). The worst performing line was the *Tub-GAL4* driven *UAS-GCLC* showing a highly significant impairment in relation to either control line as in the *Act(III)GAL4* driven experiment. This is reflected by a reduction in mean values of 36.06% relative to the driver control and 28.46% relative to the responder control; a reduction in median values of 45.10% relative to the driver control and 33.33% relative to the responder control; and a reduction in maximum lifespan of 14.52% relative to the driver control line and 7.02% relative to the responder control line (Table 3.13). The curve profile is similar to that seen in the *Act(III)GAL4* driven experiment, with a high initial mortality, followed by greater mortality acceleration relative

to the control lines. There is an even more pronounced late flattening of the survival curve than with the *Act(III)GAL4* driver but still a reduced maximum lifespan relative to the control lines.

Figure 3.15 Lifespan assay for *Tub-GAL4* driver and *UAS-GCLC* responder lines and relevant controls. Each dataset is pooled data from 3-4 replicate tubs run simultaneously. The survival plots are significantly different (Log Rank test, p=<0.0001). Pairwise comparison is detailed in Table 3.12

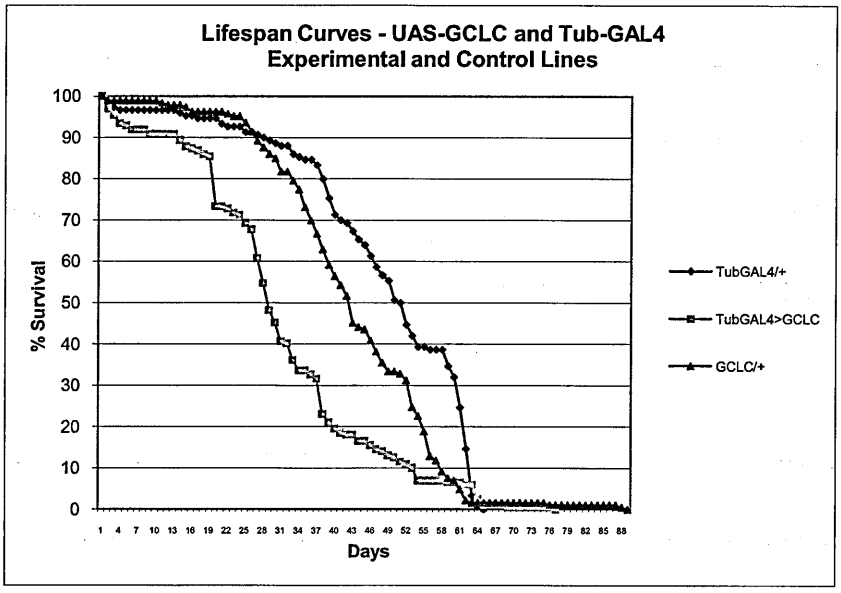


Table 3.12 Summary Statistics for Lifespan assay for *Act(III)GAL4* driver and *UAS-GCLC* experimental and control lines

Genotype	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value	DF
Tub-GAL4/+	150	47.26	1.22145	51	62	<0.0001	2
UAS-GCLC/+	186	42.24	0.97481	42	57		
Tub -GAL4>UAS-GCLC	199	30.22	1.07939	28	53		

Table 3.13 Pairwise comparison between genotypes for lifespan for *Act(III)GAL4* driver and *UAS-GCLC* experimental and control lines. In the case of both comparisons involving *Tub-GAL4* driven *UAS-GCLC* flies, the figures refer to an increase or decrease in longevity of the driven flies relative to the control flies. In the case of the comparison of driver and responder control lines, the figures refer to an increase or decrease in longevity of the responder control flies relative to the driver control flies.

Comparison	% Change (Mean)	% Change (Median)	% Change (Max 90%)	Log Rank p-value	DF
Tub -GAL4/+ vs Tub -GAL4>UAS-GCLC	↓ 36.06	↓ 45.10	↓ 14.52	<0.0001	1
UAS-GCLM/+ vs Tub -GAL4>UAS-GCLC	↓ 28.46	↓ 33.33	↓ 7.02	<0.0001	1
Tub -GAL4/+ vs UAS-GCLC/+	↓ 10.62	↓ 17.65	↓ 8.06	<0.0001	1

3.3.3 Ubiquitous Co-Over-expression of GCLM and GCLC

In vivo, GCLC and GCLM associate to form a holoenzyme complex and their expression is upregulated under conditions of oxidative stress. The holoenzyme complex is a far more catalytically efficient enzyme unit than the individual sub-units alone (see Section 1.5.2). A recombinant fly strain containing a chromosome with both the *UAS-GCLC* and *UAS-GCLM* elements was used to examine the effects of co-overexpression of both subunits. The fly strain was subsequently isogenised in a similar fashion to that described in Figure 3.2, by back-crossing to w^{1118} for a minimum of 8 generations. For each of the generational crosses, flies with the darkest eyes were chosen as this indicated the presence of 2 w^{+} genes confirming that *UAS-GCLC* and *UAS-GCLM* had not recombined away from each other leaving a line with either element singly. In order to confirm that the impairment of lifespan seen when the catalytic subunit alone was over-expressed ubiquitously was not a result of an imbalance between expression levels of GCLC and GCLM, crosses were set up as detailed in Figure 3.1 using *UAS-GCLC,GCLM* and both the actin and tubulin drivers. In both cases, no male progeny were generated for the lifespan assays and very few female progeny survived. This implies that the developmental effect seen when *UAS-GCLC* is over-expressed is more pronounced with the more efficient holoenzyme mimic, leading to high levels of pre-adult lethality.

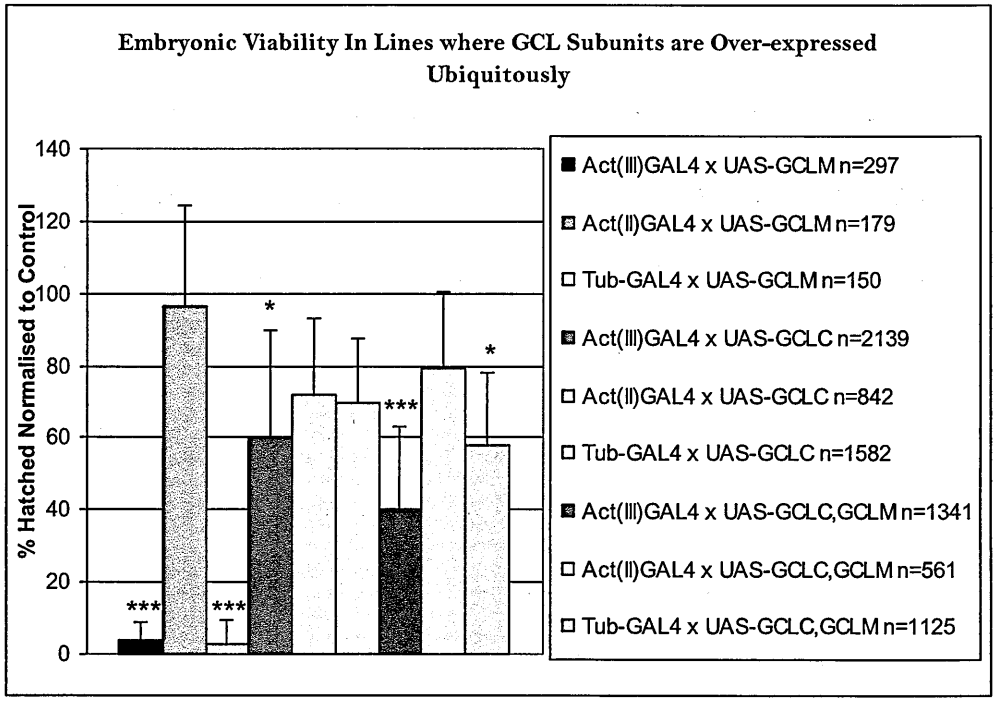
3.3.4 The Effect of GCL Sub-Unit Over-Expression on Embryonic Viability

In order to assess whether the adult lethality seen when GCLC and GCLM are co-overexpressed in a ubiquitous pattern is reflected in a reduced level of embryonic survival, *UAS-GCLM*, *UAS-GCLC* and *UAS-GCLC,GCLM* responder lines were crossed to a variety of GAL4 drivers which drive expression at high levels, in a ubiquitous pattern (Figure 3.16). If lethality during embryogenesis is responsible for the absence of one or more classes of adult progeny in these driven recombinant lines, then ubiquitously driven *UAS-GCLC,GCLM* lines would be expected to show the most severely impaired embryonic hatch numbers relative to control lines as the adult lethality phenotype was most pronounced when this line was crossed to drivers with a high-level ubiquitous expression pattern (Section 1.2.5). In addition, ubiquitously driven *UAS-GCLM* would not be expected to impair hatch rate as no obvious lethality was observed (Section 3.3.1)

Contrary to these predictions, the most extreme embryonic lethality phenotype is seen when *UAS-GCLM* is crossed to either *Act(III)GAL4* or *Tub-GAL4* (Figure 3.16). The deviation from control hatching means is highly statistically significant in both cases with less than 5% relative survival when these drivers are used. In contrast, when a second chromosome GAL4 driver (*Act(II)GAL4*) is used, there is no significant difference between heterozygous control hatching means and the *Act(II)GAL4* driven *UAS-GCLM*. This contradicts expectations based on Mendelian genetics, as even selective loss of driven flies should only lead to a reduction of 50% relative to control lines. The GAL4 driver lines

showed similar levels of expression when crossed to a *UAS-GFP* responder line (Figure 3.6) so this selective lethality is likely to be the result of some factor unrelated to the over-expression of *UAS-GCLM*. This pattern is replicated when the same drivers are used to induce over-expression of the catalytic subunit alone and both the catalytic and modifier subunits together. In each case, crosses between the responder lines and the second chromosome driver show no significant survival impairment relative to control lines whereas both *Act(III)GAL4* and *Tub-GAL4* crosses show statistically significant impairment relative to control lines when these drivers are crossed to *UAS-GCLM* and *UAS-GCLC,GCLM*, as does *Act(III)GAL4* when crossed to *UAS-GCLC*.

Figure 3.16 Assay examining the effects of ubiquitous over-expression on embryonic viability. Histogram shows embryo hatching figures for crosses using male flies from GAL4 driver lines and female flies from UAS-responder lines. These data are shown as a relative percentage of the control crosses between female flies from each UAS-responder line and w^{1118} males. In each case, significant deviation from the control hatching numbers is indicated by asterisks (* p=<0.05, ** p=<0.01, *** p=<0.001, Student's t-test). Where no asterisk is present, the difference is not statistically significant. Error bars represent standard deviation. The n value is lower for the GCLM crosses as GCLM flies lay at a lower level than the other strains and, in addition, a portion of the collection plates for this experiment had to be discarded due to a bacterial infection which could have influenced the results.



3.3.5 Effect of GCL Sub-Unit Over-expression on Adult Survival

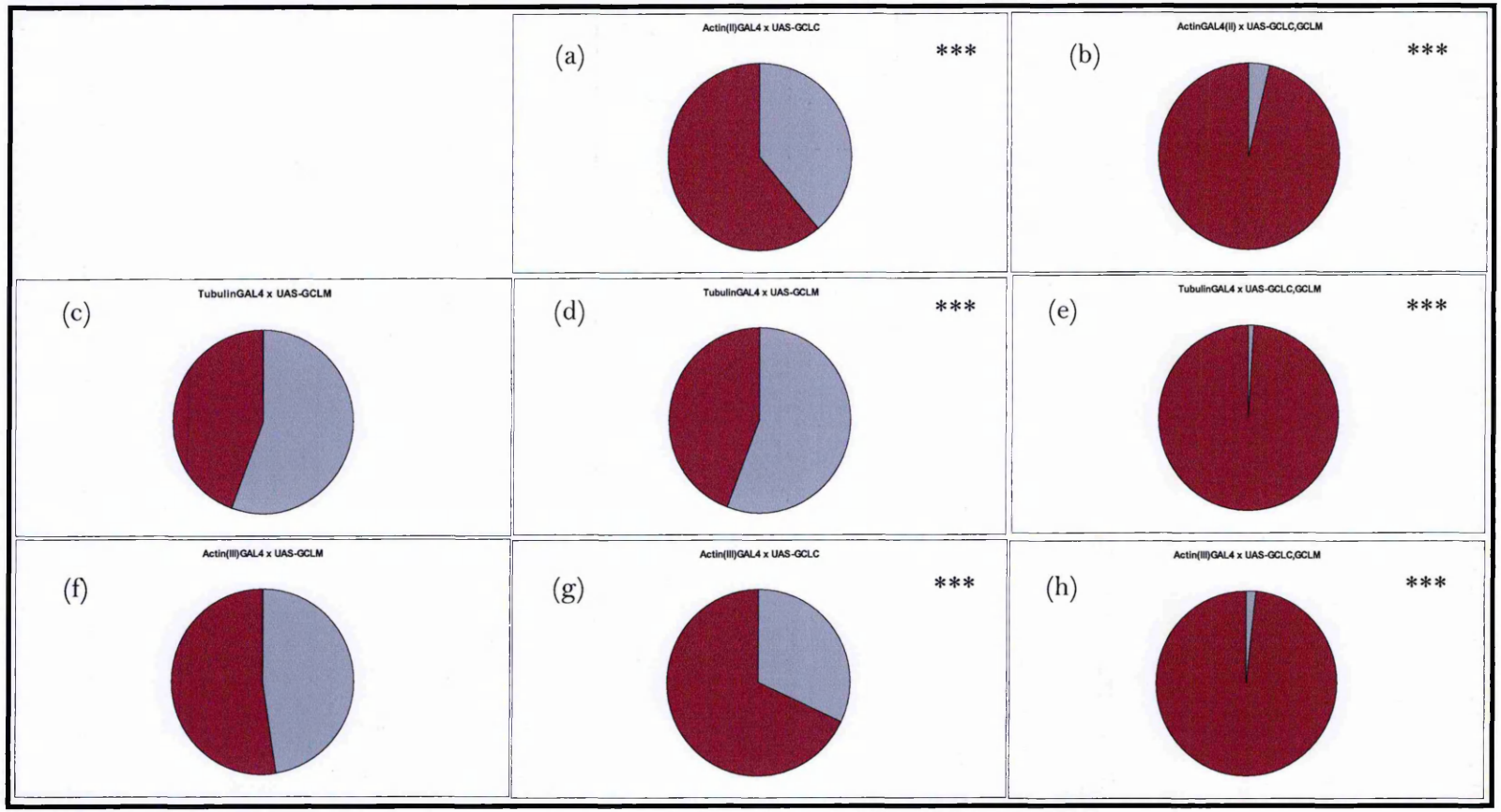
In order to quantify adult survival, progeny from crosses between the 3 ubiquitous GAL4 drivers and all three responder strains were allowed to age and eclose and the adults were scored for genotype (Figure 3.17).

Based on the embryonic viability data shown in the previous section, it can be predicted that, should the defining factor in adult survival be the same factor causing driver-specific embryonic lethality, all crosses using the *Actin(II)GAL4* driver would show a predicted 50:50 ratio between driven and non-driven flies. All crosses using the *Act(III)GAL4* driver and the *Tub-GAL4* driver would be expected to show severe lethality but no variation from the 50:50 ratio of driven and non-driven flies and there would not be any predicted variation between crosses using the *UAS-GCLM, UAS-GCLC* and *UAS-GCLC,GCLM* responder lines. As shown in Figure 3.17, this is not the case. Despite a low embryonic survival percentage when *UAS-GCLM* is crossed to either *Act(III)GAL4* or *Tub-GAL4*, there is no significant difference between the number of driven and non-driven flies that survive, supporting the hypothesis that this early lethality is unrelated to the over-expression of GCLM. However, both *UAS-GCLC* and *UAS-GCLC,GCLM*, when over-expression is driven ubiquitously, show a highly statistically significant deviation from the predicted 50:50 ratio of driven to non-driven progeny. In both cases, survival of the flies over-expressing GCLC or both GCLC and GCLM together, is highly statistically

significantly impaired in relation to non-driven sibling flies. In the case of *UAS-GCLC,GCLM*, driven flies are virtually absent among F_1 progeny.

This implies that, in addition to embryonic lethality caused by some factor external to GCL expression levels, there is also GCL over-expression dependent lethality. Bearing this in mind, it is necessary to remove the possibility of the externally confounding factor of a possible *Wolbachia* infection before examining further where the expression-dependent lethality occurs.

Figure 3.17 Comparison of surviving adult genotypes from crosses between *UAS-GCLM* ([c], [f]), *UAS-GCLC* ([a], [d], [g]) and *UAS-GCLC,GCLM* ([b], [e], [h]) and *Act(II)GAL4*, *Act(III)GAL4* and *Tubulin-GAL4* drivers. Mauve segments represent flies containing both driver and responder elements (driven flies), burgundy segments represent sibling control flies carrying only the UAS-responder elements. All data are expressed as a percentage of the total number of surviving adult flies. Significance of the deviation from the expected 50:50 ratio is indicated by asterisks (* p=<0.05, ** p=<0.01, *** p=<0.001, Chi-squared test). Where no asterisk is present, the difference is not statistically significant.



These data imply that it is possible that something additional to genotype is having an effect on embryonic survival. The intracellular bacterium *Wolbachia* is prevalent in insect species (Hilgenboecker *et al* FEMS 2008) and known to be responsible for the phenomenon of cytoplasmic incompatibility, a type of inherited reproductive failure (Laven 1959; Yen & Barr 1973; Clark et al 2005), the consequences of which are described in Figure 3.18.

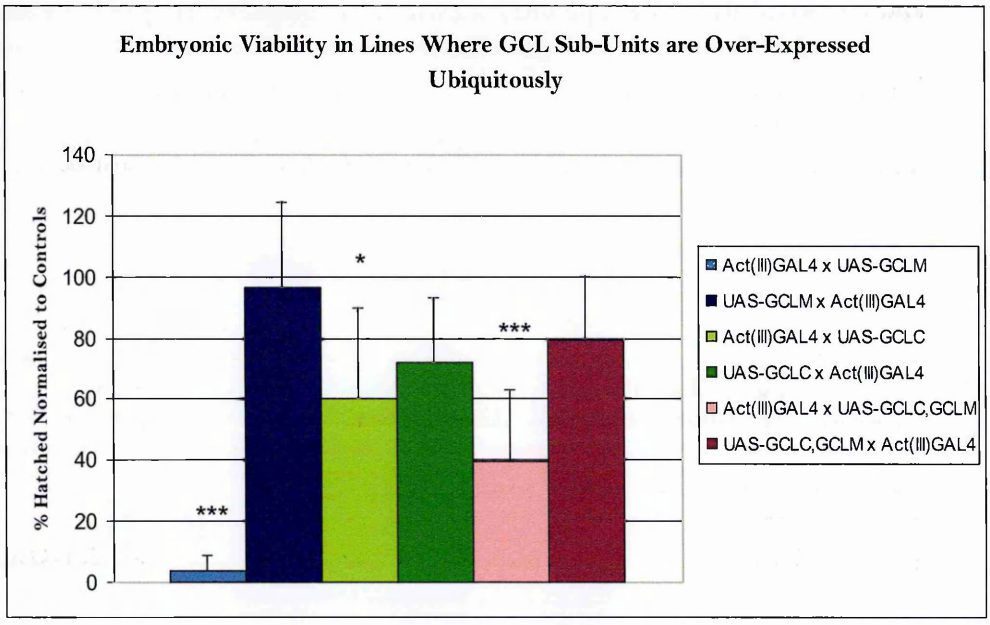
Figure 3.18 Cytoplasmic incompatibility in *Drosophila melanogaster*. The grid below shows the outcomes of the four possible mating combinations between infected and uninfected parental flies Crosses involving infected males and females, non-infected males and females and infected females and non-infected males give rise to normal development. Crosses where male flies are infected with *Wolbachia* while their female mating partners are not lead to early embryonic lethality. (Clark *et al* 2005).

	♀ Parent Uninfected	♀ **Parent Infected**
♂ Parent Uninfected	Normal Development	Normal Development
♂ **Parent Infected**	**Early Embryonic Lethality**	Normal Development

It has been reported that *Wolbachia* infection is present in approximately 30% of stocks held at the Bloomington stock centre from which the driver lines used in these assays were acquired (Clark *et* al 2005). Therefore, it is possible that a *Wolbachia* infection of the driver lines could lead to the embryonic lethality phenotype shown in Figure 3.15. If the two driver lines used in this assay are infected with *Wolbachia*, it would be predicted that a reversal of the cross direction, with male parental flies coming from the UAS-responder lines and female flies from the GAL4 driver lines, would lead to a reversal of the lethality phenotype. As shown in Figure 3.19, this is the case. For each cross, mean hatching numbers show no significant difference from control hatching numbers when the cross

direction is reversed. All fly strains were, therefore, treated for *Wolbachia* infection, as described in Section 1.2.2 (Methods and Materials) and all data from Section 3.3.6 onwards refers to experiments carried out using uninfected lines.

Figure 3.19 Assay examining the effect of cross direction on embryonic viability when GCL subunits are over-expressed ubiquitously. In the figure legend, the first strain listed in each cross is the male parent. These data are shown as a relative percentage of the control crosses between responder lines and w^{1118} flies. In each case, significant deviation from the control hatching means is indicated by asterisks (* p=<0.05, ** p=<0.01, *** p=<0.001, Student's t-test). Where no asterisk is present, the difference is not statistically significant. Error bars represent standard deviation.



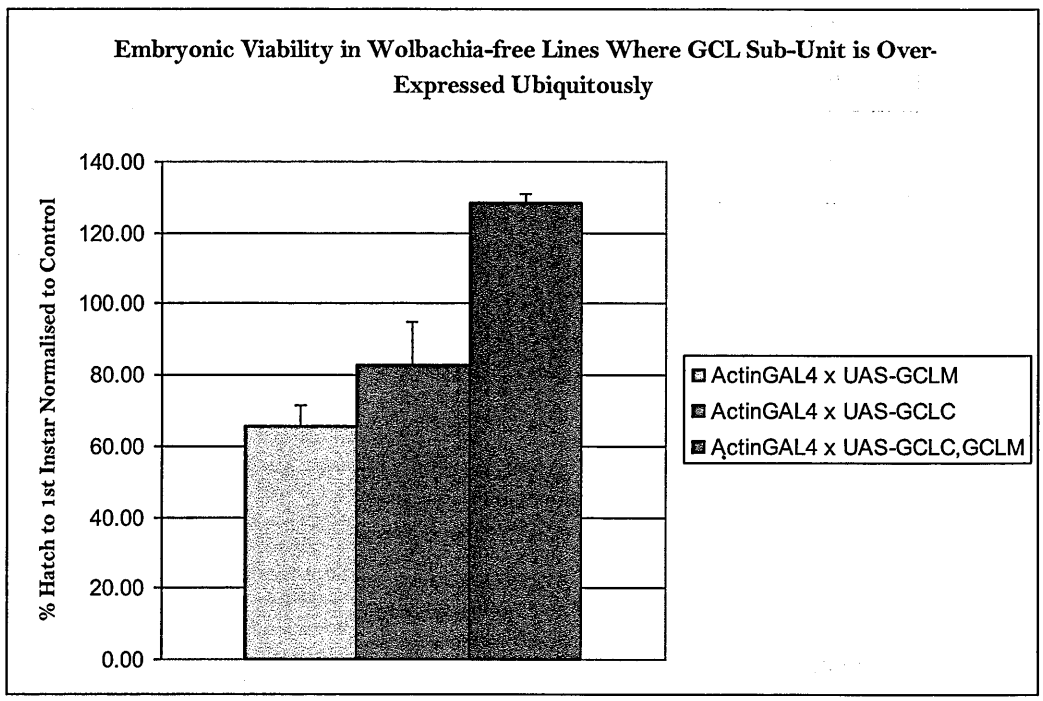
3.3.6 Isolating the Developmental Stage Where Lethality Occurs

To enable effective identification of F_1 genotypes throughout development and larval morphogenesis, two driver lines were made containing balancer chromosomes with easily distinguishable phenotypes (See Figure 3.2 and Figure 3.3).

When embryonic viability was assayed in lines that had been passed through a preventative antibiotic protocol to remove any *Wolbachia* infection present, ubiquitous over-expression

of any combination of GCL subunits did not lead to embryonic lethality (Figure 3.20). F_1 progeny are virtually absent from all crosses between the *UAS-GCLC,GCLM* responder line and the ubiquitous GAL4 drivers used in this assay. If this lethality was embryonic, there would be a massive mortality event relative to controls at this stage. In fact, the opposite is true, with *Act(II)GAL4 (CyO, GFP)* and *UAS-GCLC,GCLM* crosses showing a non-significant increase in hatching numbers relative to the control cross. Whilst *UAS-GCLM* and *UAS-GCLC* do show a slight reduction in embryonic viability relative to controls, this is not significant. Therefore, after removal of the *Wolbachia* infection, all genotypes show normal hatch rates relative to control lines and any lethality seen must be a result of events that occur after hatching.

Figure 3.20 Assay examining the effects of ubiquitous over-expression on embryonic viability. Histograms show embryo hatching figures for crosses using male flies from the Act(II)GAL4 (CyO, GFP) driver strain and female flies from the UAS-responder strains. The data are shown as a relative percentage of the control crosses between females from each UAS-responder line and w^{1118} males. There was no significant difference between experimental and control hatching means (Student's t-test). Error bars show standard deviation.



As significant genotype-specific lethality is not present during embryogenesis but adults show severely reduced genotype-specific survival, it follows that a lethality event occurs at some point during larval development or pupation. In order to ascertain the lethal phase, crosses were set up using actin-GAL4 and tubulin-GAL4 drivers, according to the protocol described in Section 2.3.7 Methods and Materials. The results are shown in Figure 3.21.

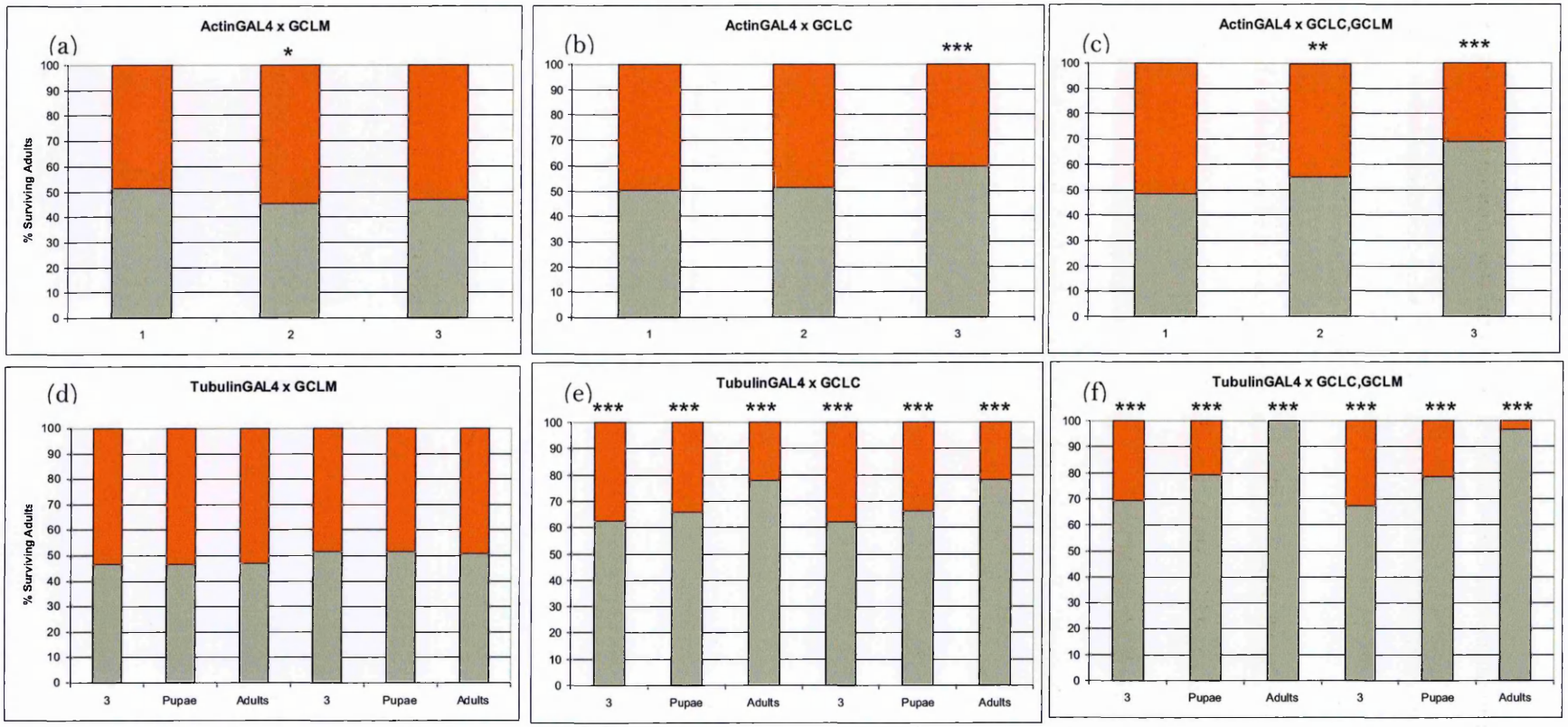
As expected, crosses between *UAS-GCLM* and *Act(II)GAL4 (CyO, GFP)* and *Tubulin-GAL4 (Sb Tb)* show no significant deviation from the expected 50:50 adult sibling genotype distribution. This is independent of driver used or the direction of the parental cross (i.e. whether the driver line was maternal or paternal). There is one anomalous significant deviation at 2nd instar when *UAS-GCLM* is crossed to *Act(II)GAL4 (CyO, GFP)* but this is an enrichment of driven flies rather than a reduction. While there is no explanation for this, it could merely be a chance occurrence and does not appear to be related specifically to GCLM over-expression as no subsequent increase is seen in 3rd instar driven survival nor is this replicated in the crosses using the *Tub-GAL4 (Sb Tb)* driver.

The situation is different for *UAS-GCLC*. Figure 3.21 panel [b] shows that for 1st and 2nd instar larvae, as with *UAS-GCLM* flies, there is no significant difference between the survival of flies over-expressing GCLC and those that are not. However, by 3rd instar, a highly statistically significant depletion of driven flies is seen in the surviving progeny (Figure 3.21, panel [b]). This depletion continues, becoming more pronounced at each time point, throughout 3rd instar, pupal and adult phases (Figure 3.21, panel [e]). It is

clear that when *UAS-GCLC* is over-expressed ubiquitously throughout development, the population progressively loses individuals where over-expression occurs.

This lethality effect is earlier and more pronounced in crosses where GCLC and GCLM are co-overexpressed, where a highly statistically significant depletion of driven flies at 2nd instar (Figure 3.21, panel [c]) and a highly statistically significant effect at 3rd instar and pupation are observed. By adulthood, only rare individuals bearing both the responder and driver elements are recovered (Figure 3.21, panel [f]).

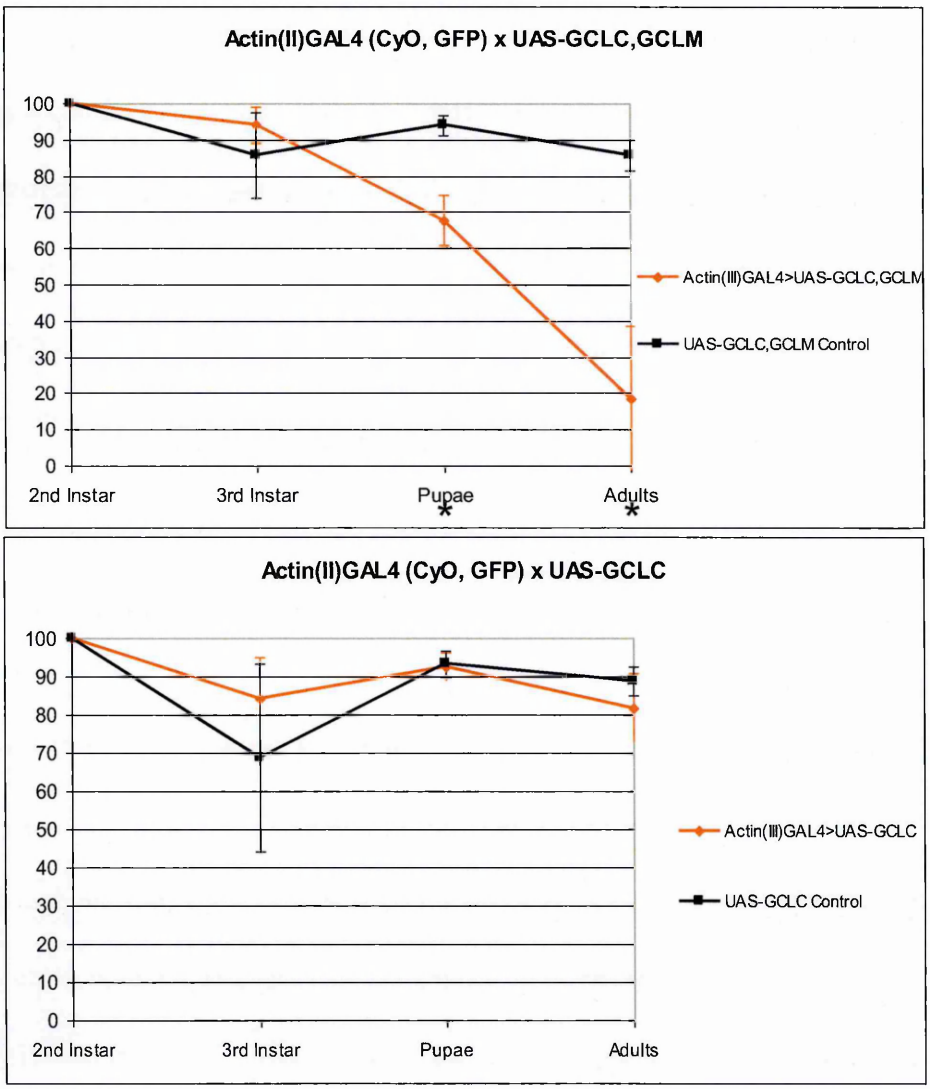
Figure 3.21 Assay examining genotype ratio of surviving 1st, 2nd and 3rd instar larvae ([a], [b], [c]) and 3rd instar larvae, pupae and adults ([d]. [e], [f]) when UAS-GCLM ([a],[d]), UAS-GCLC ([b], [e]) and UAS-GCLC,GCLM ([c], [f]) are over-expressed ubiquitously throughout development. Red bars indicate percentage of total individuals surviving where both driver and responder elements are present (i.e. driven); grey bars indicate percentage of total individuals surviving where only the responder element is present (i.e. non-driven siblings). Significance is indicated by asterisks (* p=<0.05, ** p=<0.01, *** p=<0.001, Chi-squared test). In panels [a]-[c], male parental flies came from the driver strain. In panels [d]-[f], the first three columns in each panel represent crosses where male parental flies came from the driver strain, the last 3 columns represent crosses where male parental flies came from the responder strain.



In order to ascertain whether this effect was a cumulative effect, with significant mortality events during each developmental phase or whether it was a single extreme event leading to early mortality and selective depletion of driven flies which then affected survival figures for later lifecycle phases, a single population of larvae were followed through from 2nd instar to adulthood and their survival noted at each stage. The results are shown in Figure 3.22. Each data point represents the percentage of surviving individuals expressed as a percentage of the total number of surviving individuals at the previous phase of development, hence allowing the isolation of phases where mortality was high. Due to the invasive nature of the harvesting and transplantation of larvae and the necessity for UV exposure to detect GFP expression during the initial collection, the earliest larval phase collected and scored was 2nd instar. This was decided upon as a result of pilot work that indicated a high universal mortality due to experimental manipulation in 1st instar larvae.

For *UAS-GCLC,* there was no significant difference between survival of driven and non-driven flies at any point during development, despite the reduction in absolute survival numbers for driven flies shown in the previous section. It is possible that the significant differences shown previously were masked by intra-replicate variation in this assay – each replicate consisted of a single vial seeded with 20 larvae. However, *Actin(II)GAL4 (GFP, CyO)* driven *UAS-GCLC,GCLM* flies did show significant lethality. This was not restricted to a single developmental phase but occurred both during 3rd instar and during pupation.

Figure 3.22 Assay following specific population of flies through from 2nd instar to adulthood. Red lines represent flies carrying both the GAL4 driver and UAS-responder chromosome (i.e. driven). Black lines represent flies carrying only the UAS-responder chromosome (i.e. non-driven). Each point shows surviving individuals as a percentage of the total number of individuals scored as surviving at the previous developmental stage. The significance of the difference between the mean experimental survival (i.e. driven) and the mean control survival (i.e. non-driven) is indicated by asterisks (* p=<0.05, ** p=<0.01, *** p=<0.001, Student's t-test). Where no asterisk is present, the difference is not statistically significant. Error bars represent standard deviation.

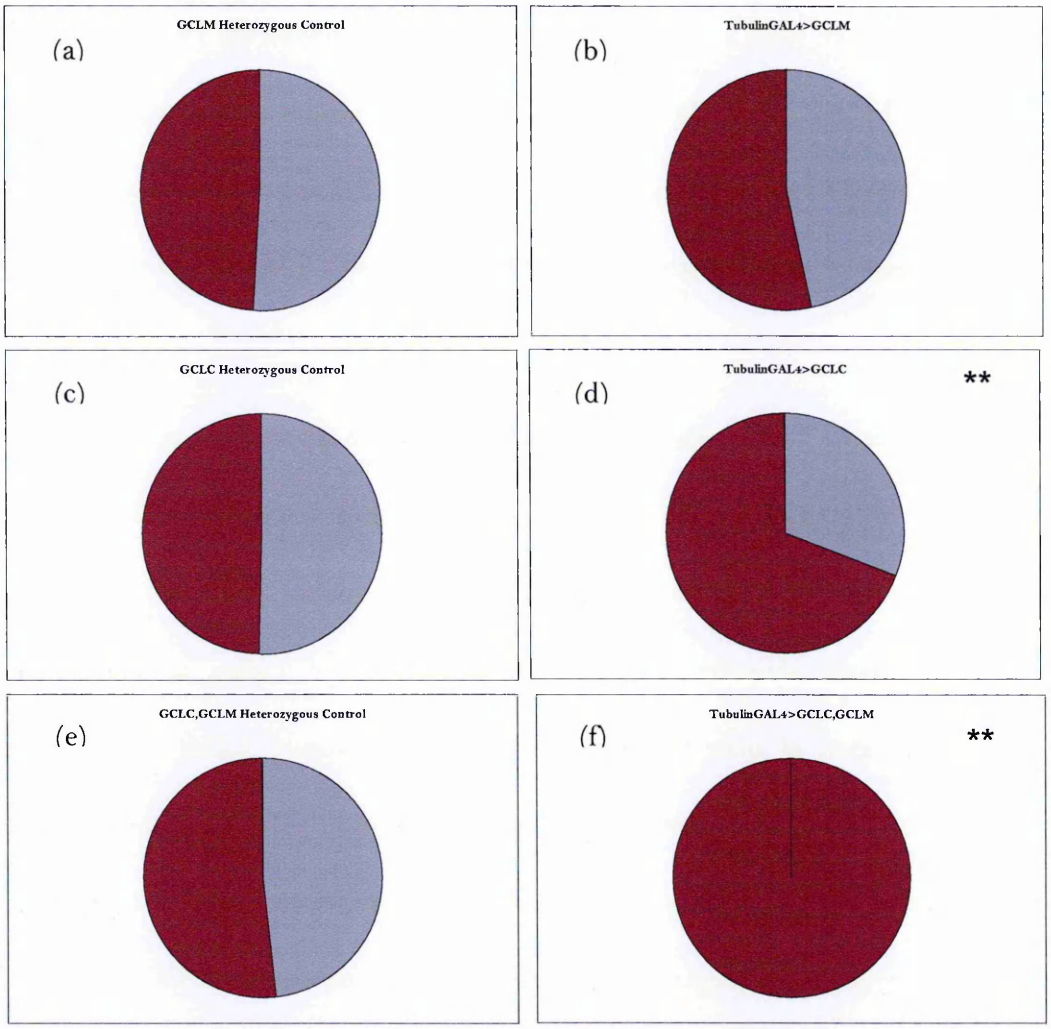


3.3.7 Effect of GCL Sub-Unit Over-Expression on Adult Sex Ratio

Under normal circumstances, the sex ratio of adults will be 1:1 male to female flies. However, when all surviving adults from crosses between the *Tub-GAL4 (Sb Tb)* driver flies and *UAS-GCLM, UAS-GCLC* and *UAS-GCLC,GCLM* responder strains are scored for sex, there is a genotype-specific deviation from this 1:1 ratio when the catalytic subunit is over-expressed either alone or in conjunction with the GCLM modifier subunit (Figure 3.23).

When GCLM is over-expressed ubiquitously, the expected 1:1 ratio of male to female flies is maintained in both the driven flies and their heterozygous UAS-GCLM non-driven siblings (Figure 3.23a and Figure 3.23b). In contrast, when the catalytic subunit is over-expressed ubiquitously, this 1:1 ratio is disturbed leading to a statistically significant depletion of male flies in surviving progeny (Figure 3.23d) with females comprising 69.12% of the surviving flies; in heterozygous *UAS-GCLC* control sibling flies, the expected 1:1 ratio of male to female flies is maintained (Figure 3.23c). This depletion of surviving male progeny is more pronounced in crosses where GCLC and GCLM are co-overexpressed ubiquitously, when all surviving driven flies are female. Thus, elevation of GCLC leads to selective loss of male progeny.

Figure 3.23 Comparison of sex of surviving adult flies from crosses between *UAS-GCLM* ([a], [b]), *UAS-GCLC* ([c], [d]) and *UAS-GCLC,GCLM* ([e], [f]) and *Tub-GAL4 (Tb Sb)* driver. In each case, each pair of panels represents siblings from a single cross between male driver and female responder element flies. These siblings were divided according to genotype and scored for sex. Significant differences from an expected 50:50 sex ratio are shown by asterisks (* p=<0.05, ** p=<0.01, *** p=<0.001, Chi-squared test). Where no asterisk is present, the difference is not statistically significant. Burgundy segments represent female flies and mauve segments represent male flies.



3.4. Discussion

3.4.1 GCLM

Taken together, the results of over-expression of GCL subunits driven by actin and tubulin promoters do not support the hypothesis that over-expression of GCLM is beneficial to an organism's lifespan. Neither do they indicate that it is detrimental. These results were predicted based on the fact that GCLM has no demonstrable catalytic activity in isolation and would therefore be unlikely to be beneficial to an organism in excess in the absence of a commensurate increase in the levels of GCLC, the catalytic subunit with which it combines to form a catalytically efficient holoenzyme complex. Despite the attempted isogenisation of the fly strains used in this assay, genetic background is still highly influential, as demonstrated by the results of the *Tub-GAL4* driver experiment, with the main defining factor being the presence or absence of the driver chromosome. This highlights the need for an experimental design that minimises or preferably eliminates background genetic variation between experimental and control lines.

These results contradict those published by Orr *et al* in 2005 (Orr, Radyuk et al. 2005). Orr and colleagues reported that global over-expression of GCLM using a Tub-GAL4 driver led to statistically significant increases in mean lifespan of up to 24% relative to both heterozygous driver and responder element controls (Orr, Radyuk et al. 2005). This was replicated over 3 independent insertion responder lines generated by P-element

remobilisation. In addition, both experimental and control lines are longer lived under experimental lifespan conditions than those of our lab.

There could be several explanations for these differences. The gross longevity differences between strains could be a result of a difference in the genetic background of each strain; the *Tub-GAL4* driver used in our experiments was a different driver to that used by Orr and colleagues and our UAS-responder elements were made in this laboratory. In addition, the experimental procedures differed between the two laboratories, with our lifespan experiments being carried out in population tubs rather than vials greatly increasing the flies' activity over the course of their lifespan. This could conceivably have an effect on the longevity of flies in comparison to the less active environment created by confining flies to a smaller vial. Population density was very different in the experiments described by Orr and colleagues, whose flies were kept at a volume of 20 per vial in comparison to the numbers detailed in the lifespan work presented here. Nonetheless, as has been observed when analysing lifespan studies investigating the effect of over-expression of SOD in *Drosophila* (Orr and Sohal 2003), it is flies with a reduced control lifespan that tend to show the greatest increases in longevity when components of the antioxidant defence system are over-expressed, therefore these data are contrary to that prediction.

It is conceivable that the GCLM P-element insertion used in our experiments is exerting some kind of position effect that is detrimental to the survival of the strain, impacting the function of an unrelated gene and that this is partially ameliorated by the benefit afforded

by the presence of the Tub-GAL4 chromosome in experimental crosses. In order to fully examine this, it would be necessary to remobilise this element and test a variety of insertion lines. Whilst evidence of extension across multiple experiments from multiple laboratories maintaining flies under differing conditions could be seen as evidence of a 'real' effect, the fact that this is not the case here does not necessarily imply that the effect seen by Orr and colleagues is not significant. The fact that the presence of the *Tub-GAL4* driver chromosome has such an overwhelming effect on the results presented above could mask any smaller increases in lifespan that may be due to GCLM over-expression. However, the data presented above indicate that when a second high-level global driver is used (*Act(III)GAL4*), one which does not have the complicating effects of increased control lifespan relative to experimental and GCLM heterozygous control lines, there is still no significant increase in mean longevity when GCLM is over-expressed. It is possible that the small but replicable increase in lifespan seen by Orr and colleagues (2005) is modest enough to be affected by subtle differences in culture conditions and experimental conditions between laboratories.

3.4.2 GCLC and The Holoenzyme Complex

Contrary to the original hypothesis that over-expressing GCL in *Drosophila* would lead to increased longevity as a result of a reduction in oxidative damage, these data actually show that high-level global over-expression is detrimental to survival. This concurs with the results published by Orr and colleagues, where *UAS-GCLC* over-expression reduced

lifespan in 2 out of 3 independent insertion lines (Orr, Radyuk et al. 2005). Whilst nothing can be deduced about the role of oxidative stress in ageing from this reduction in lifespan, it is very interesting from the perspective of considering the effects of perturbation of redox-state during larval development and pupation. The fact that there appears to be non-age-dependent mortality in flies where the catalytic subunit of the rate-limiting enzyme in the glutathione synthesis pathway is over-expressed, implies that an increase in glutathione titre when present throughout development is in some way detrimental to fitness on eclosion. This is further supported by the severe reduction in progeny when the recombinant *UAS-GCLC, GCLM* holoenzyme mimic is over-expressed throughout development. It is possible that extremely high glutathione titre resulting from the over-expression of a more efficient holoenzyme mimic lead to this most extreme phenotype. A similar lethal phenotype is seen when SOD is over-expressed throughout development (Seto, Hayashi et al. 1990) further supporting the hypothesis that extreme perturbation of redox balance has a developmental consequence in *Drosophila.* This is relevant to further analyses as it must be borne in mind that over-expression in any tissue during development may have effects that carry through to the adult fly. Ideally, to isolate effects in adulthood from developmental effects, over-expression should be induced post-eclosion.

High-level global over-expression is a non-specific pattern involving all tissue types. It has been suggested that the individual tissue type where over-expression is induced may be crucial in extending lifespan (Orr and Sohal 1993; Parkes, Elia et al. 1998; Orr, Mockett et al. 2003; Orr, Radyuk et al. 2005; Luchak, Prabhudesai et al. 2007). As Orr *et al* demonstrate position effect of the P-element insertions can have implications for survival

(Orr, Radyuk et al. 2005). All data presented here are from single insertion lines. Ideally, these should be remobilised and individual lines assessed. Nonetheless, the concurrence between these data and the results published by Orr *et al* support the conclusions drawn. These data raise interesting questions about the role of redox balance in larval development, especially in the context of the role of ROS in signalling and proliferative/apoptotic pathways.

Over-expression of both GCL subunits ubiquitously throughout development has two consequences:

(i) Lethality from 3rd larval instar onwards leading to exceedingly low adult survival

(ii) Selective depletion of surviving adult male flies relative to surviving female flies.

The second phenotype is seen at a reduced level in flies over-expressing the catalytic subunit alone. When GCLM is over-expressed in isolation, there is no lethality, nor is there any deviation from expected ratios. Despite their apparent association, it is necessary to consider both these as separate phenotypic effects.

3.4.3 Lethality

Late instar lethality is more pronounced in the recombinant line where both sub-units are over-expressed. This makes is difficult to measure glutathione levels as many larvae do not survive through to pupation and eclosion of adult flies. It would be informative, as future

work, to measure glutathione levels in surviving larvae and in first and second instar larvae. However, these values would also have to be interpreted with caution as individual larvae that survive may survive *because* they express GCL at lower levels than their dead siblings and there is no way to verify this by comparing with the individuals that have died prior to this stage. Nonetheless, it can be inferred that co-overexpressing both subunits would lead to higher levels of GCL and hence higher glutathione titres in the larvae which do not survive. There are two possible explanations for the lethality seen in these over-expressing lines. It is possible that something specific happens from 3rd instar onwards that is particularly sensitive to perturbation of the redox status of these larvae. This is unlikely as progressive lethality is seen from 3rd instar onwards, through pupation to adulthood. Morphogenesis in *Drosophila* is triggered by a large ecdysone pulse just prior to pupation. If the redox balance affected this, it would be expected that pupation would be disrupted but that 3rd instar larval survival would not. In addition, larval moulting which occurs between 1st and 2nd and 2nd and 3rd instar larval stages is also controlled by the ecdysone signalling pathway. If this pathway is particularly redox sensitive, then there should be lethality throughout larval phases rather than from 3rd instar onwards.

A second, more likely explanation for this lethality is that a perturbation of the redox balance during larval development leads to a gradual accumulation of damage which reaches a critical level from 3rd instar onwards in recombinant flies, leading to high lethality. Over-expression of the catalytic subunit alone, whilst known to increase GCLC levels and glutathione titres, may do so at a lower level than when both the catalytic and modifier subunit are over-expressed as it is known that GCLC is less catalytically efficient in the

absence of GCLM (Fraser, Saunders et al. 2002). A study where SOD1 was over-expressed throughout development reported pupal lethality and increased lipofuscin levels (Seto, Hayashi et al. 1990). A gradual accumulation of damage would explain the late larval/pupal lethality timing.

Further work is now necessary to elucidate this lethality phenotype. It would be interesting to assay known oxidative damage markers such as acontinase in all larval instars and pupae. It is possible that, whilst the critical level of such damage is not reached until 3rd instar, early instar larvae still show an increase in such damage markers in relation to their sibling controls. It would also be interesting to use a ubiquitous high-level Geneswitch driver to induce expression later in development in order to test the hypothesis that it is accumulation of damage over time that causes the lethality seen here.

3.4.4 Sex Determination

The near-total absence of male progeny where both sub-units are co-overexpressed ubiquitously and the significant reduction in male progeny where GCLC is over-expressed alone, suggest that manipulating GCL levels has a profound impact on sex-specific survival. There are two possible ways in which a single sex can be under-represented in *Drosophila*:

(i) There could be selective sex-specific lethality during larval morphogenesis

(ii) The sex-ratio could be altered at the point of sex-determination during embryogenesis

The most likely process to be affected by perturbation of the redox balance is the sex determination pathway. Sex determination in *Drosophila* is under the control of *Sex-lethal* (*Sxl*) (González, Lu et al. 2008). The co-ordinate regulation of two *Sxl* promoters controls the establishment of the correct splicing pattern. This is dose-dependent, relying on the female dose of two X chromosomes to produce a *Sxl* pulse and is highly time-window dependent (González, Lu et al. 2008). Male depletion is seen in cases where a delay in embryogenesis occurs leading to accumulation of the X chromosomal product. If perturbation of the redox balance during embryogenesis leads to delayed passage through embryogeneis, this could result in feminisation of the F1 generation, as is seen here. Further work is necessary to examine whether this is the case, ideally using real-time live imaging to follow embryos through embryogenesis.

4. EFFECT OF MANIPULATION OF GCL EXPRESSION IN MOTOR NEURONS ON LIFESPAN IN *DROSOPHILA MELANOGASTER*

4.1. Introduction

Despite the fact that high-level, ubiquitous over-expression of GCL leads to impaired lifespan and lethality, it is still possible that manipulating GCL levels in a more tissue-specific manner could have a positive impact on longevity in *Drosophila.* In the last decade, it has been suggested that rather than global oxidative balance providing the key to increased longevity as a result of improved oxidative defence, it is protection from oxidative damage in specific tissues that is important (Parkes, Elia et al. 1998; Parkes, Hilliker et al. 1999; Orr, Radyuk et al. 2005; Luchak, Prabhudesai et al. 2007). These tissues are often referred to as 'life-span limiting' (Orr, Radyuk et al. 2005). Chronic and unrepaired oxidative damage to motor neurons has been proposed as a significant causative factor in organismal ageing (Parkes, Elia et al. 1998) based upon the loss of motor neurons in the brain and spinal cord in the disease Familial Amyotrophic Lateral Sclerosis (FALS), a paralytic disease that causes premature death (Parkes, Elia et al. 1998). However, FALS is a disease state and therefore may not be representative of events that occur in 'normal' ageing.

In *Drosophila,* over-expression of SOD1 and GCLC in motor neurons has been demonstrated to result in significant lifespan extension (Parkes, Elia et al. 1998; Orr,

Radyuk et al. 2005), whilst motor neuron-specific over-expression of GCLM does not (Orr, Radyuk et al. 2005). Our laboratory's UAS-responder strains have been shown to behave differently to those used in the assays carried out by Orr and colleagues, therefore, in order to assess the affect of over-expression of GCL subunits in *Drosophila* motor neurons, it was first necessary to examine whether it was possible to replicate the significant extension reported when GCLC was over-expressed in these tissues. In addition, the ratio of levels of both enzymatic subunits may be closer to that seen *in vivo* if both subunits are co-overexpressed in a fly strain containing a recombinant chromosome with both *UAS-GCLC* and *UAS-GCLM* present.

4.2. Fly Strains and Procedures

4.2.1 Fly Strains

The fly strains used in these experiments are detailed in Table 4.1 alongside the abbreviations that have been used in this chapter.

Table 4.1 Fly strains referred to in this chapter (see Section 2.1 Methods & Materials for full details of each strain)

Fly Strain	Abbreviation	P-Element Chromosome	Description
w^{1118};p{UAST GCLC T2.1.3 w^+}	UAS-GCLC	II	Containing *Drosophila* GCL transgenes in pUAST vector
w^{1118};p{UAST GCLC T2.1.3 w^+}, p{UAST GCLM T7.3.1 w^+}	UAS-GCLC,GCLM	II	
w^{1118}	w^{1118}		Laboratory reference strain
w^-; ; P{D42-GAL4}	D42-GAL4	III	Expression in motor neurons

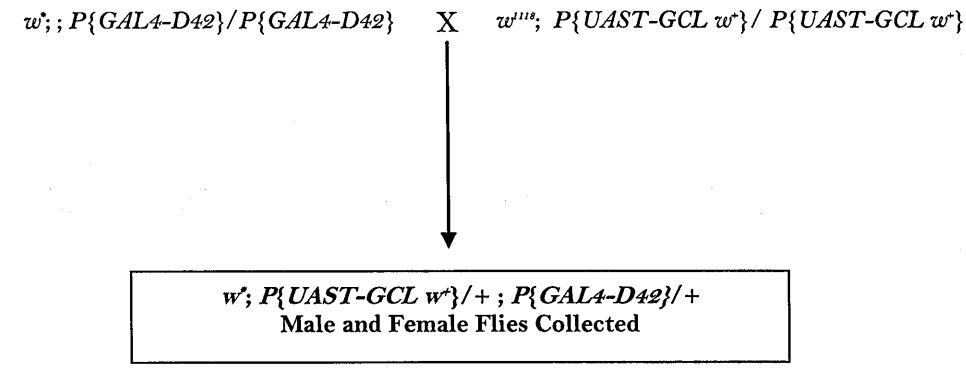
4.2.2 Procedures

Generation of Experimental Lines

In order to investigate whether tissue-limited over-expression of either the GCL catalytic subunit alone (*UAS-GCLC*) or both catalytic and modifier subunits together in a recombinant fly strain (*UAS-GCLC,GCLM*) altered longevity, flies were generated using the crossing scheme in Figure 4.1 below. The driver selected, *D42-GAL4*, drives expression in the motor neurons during larval development and adulthood. This was verified by crossing with a *UAS-GFP* stock (Figure 4.3). Female flies from both the responder lines were crossed to the *D42-GAL4* driver and both male and female flies were collected for lifespan assays. In all cases, over-expression is activated in a background in which GCLC

and GCLM are expressed from endogenous *Gclc* and *Gclm* loci; the transgenes therefore yield expression above endogenous levels.

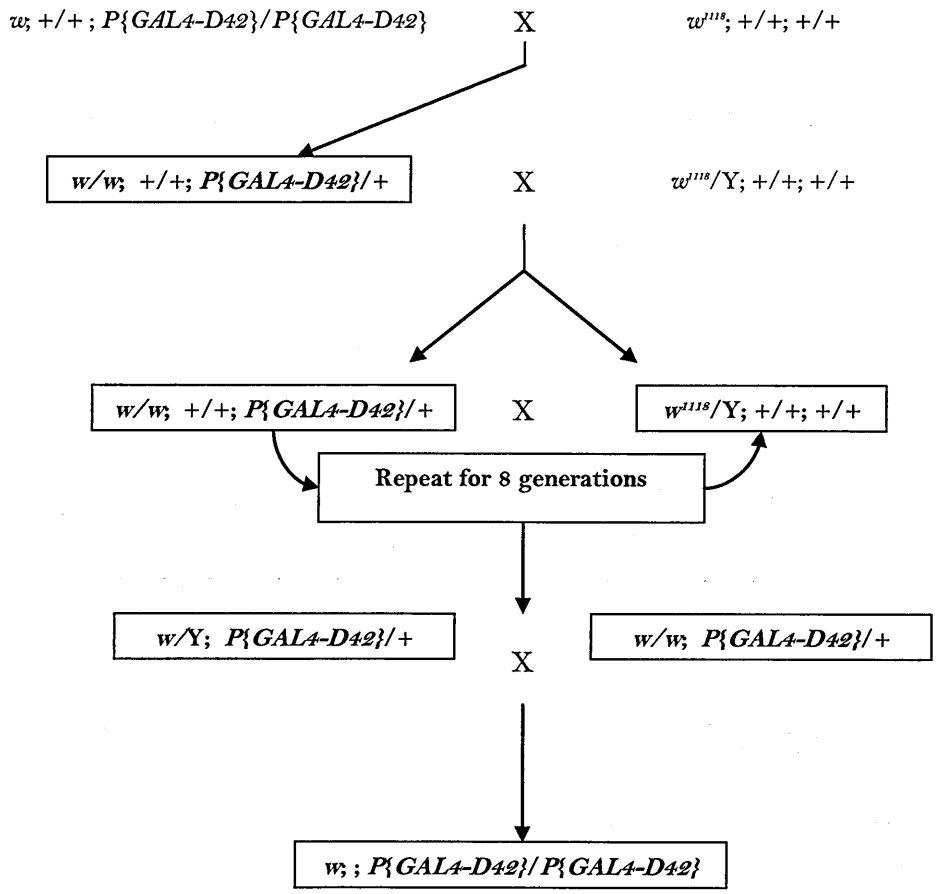
Figure 4.1 Crossing scheme used to generate flies for *D42-GAL4* driven *UAS-GCL* responder element lifespan



Isogenisation of Fly Lines

As discussed previously in Section 1.7.5, the genetic background of fly lines can have a significant effect on longevity, potentially confounding investigation of the role of single genes. In order to minimise the background differences between the experimental lines and the control lines in these experiments, these strains were isogenised by back-crossing the w^{1118}, our laboratory reference strain, for a minimum of 8 generations. The responder lines were backcrossed as described previously in Section 3.2.2, Figure 3.2. The *D42-GAL4* driver strain was backcrossed as shown in Figure 4.2 below.

Figure 4.2 Crossing scheme used to isogenise *D42-GAL4* driver flies for lifespan assays. Boxed genotypes are progeny collected from the preceding cross. Other genotypes are flies collected from main stock strain.



Lifespan Assays

Lifespan assays were carried out according to the protocol described in Section 2.3.1 (Methods and Materials) for male flies. This protocol was adapted for use with female flies, which were aged for 5 days together with male flies before being anaesthetised and placed in tubs. This was done to minimise the risk of intra-tub variation due to the presence of both virgin and non-virgin flies. The female flies were only exposed to anaesthesia once, at the time of collection and placement in the lifespan tubs. Parental crosses to generate the flies for this experiment were very productive and it was therefore possible to include a greater number of flies, synchronous in age, than in the experiments detailed in Chapter 3.

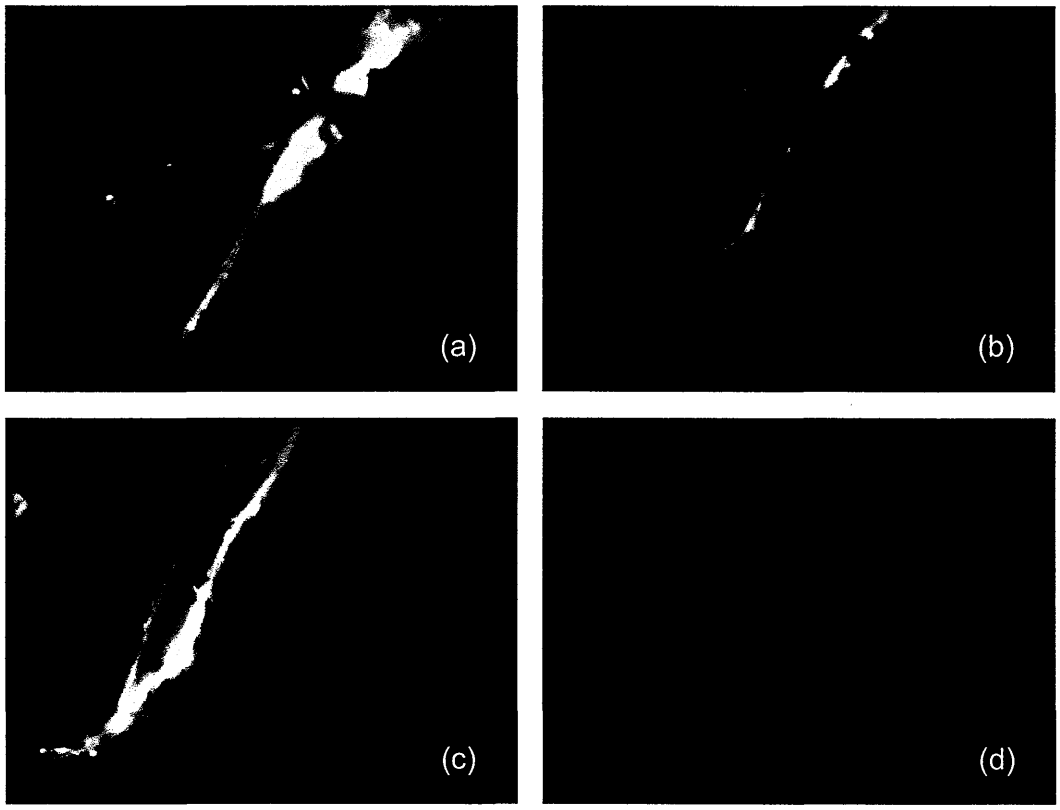
Statistical Analyses

All lifespan assays were analysed as described in Section 2.3.4 (Methods and Materials).

Verification of Driver Expression Pattern

Initially, the *D42-GAL4* driver expression pattern was verified by crossing the *w;P{GAL4-D42}/P{GAL4-D42}* strain to flies of the genotype *w*/ P{w*$^{+mC}$*=UAS-GFP S65T}T2.* The resulting progeny were then examined under a fluorescent microscope. The results are shown in Figure 4.3.

Figure 4.3 Verification of *D42-GAL4* driver expression pattern. Legs from flies of genotype *w*/ P{w^{+mC}=UAS-GFP S65T}T2/+; P{GAL4-D42/+* (Panels (a), (b) and (c)) and *w*/ + P{w^{+mC}=UAS-GFP S65T /+; +/+* (Panel (d) imaged using fluorescent microscopy (Images courtesy of P. Kansagra, personal communication).



4.3. Results

4.3.1 Overexpression of GCLC in motor neurons

Male Flies

Initially, individual replicates of each genotype were analysed to provide a statistical analysis of the spread of the replicate curves (Figure 4.4). The data shown are from several replicates of approximately 100 flies per tub run simultaneously under identical conditions and fed on media from the same batches (Figure 4.4). Within this dataset, a significant difference was observed between the individual replicates (Table 4.2). Nonetheless, as a result of the uniformity of experimental conditions and timing, these data were treated as a single set for the purpose of this analysis.

Figure 4.4 Individual replicates for lifespan assay for male flies from *D42-GAL4* driver and *UAS-GCLC* responder line. Each replicate consists of an individual tub.

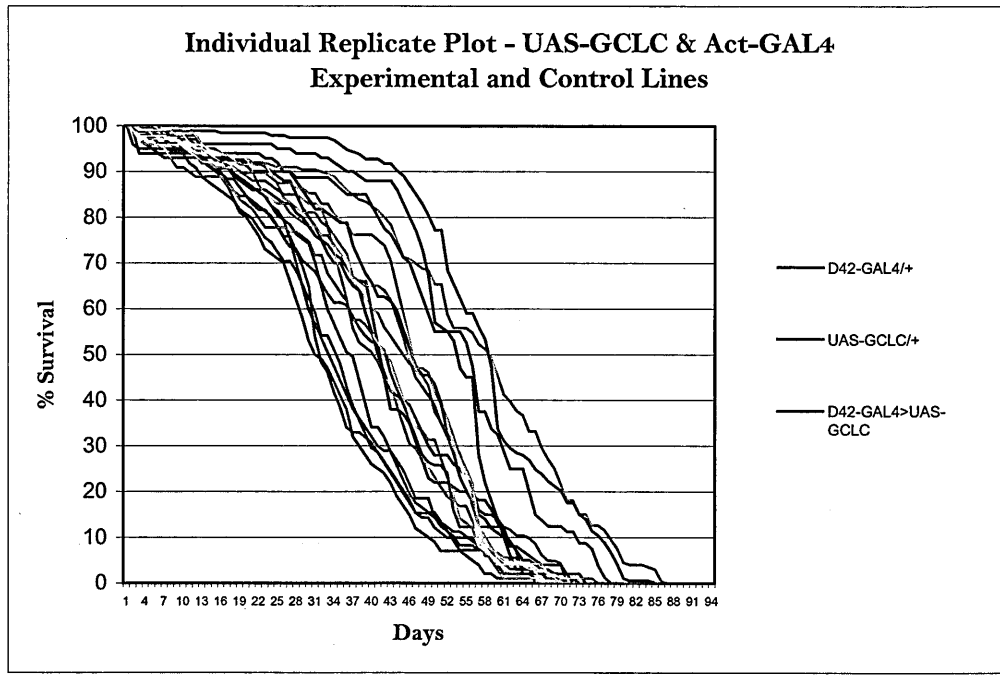


Table 4.2 Summary statistics for lifespan for individual replicates from lifespan assay for male flies from *D42-GAL4* driver and *UAS-GCLC* responder lines (experimental and control genotypes)

Genotype	Replicate	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value
D42-GAL4>UAS-GCLC	1	199	54.69	1.30544	58	77	<0.0001
	2	95	39.37	1.47226	40	57	
	3	100	41.9	1.56389	44	57	
	4	100	42.24	1.4345	45	57	
	5	88	43.65	1.57734	46	58	
	6	99	37.77	1.63412	42	56	
D42-GAL4/+	1	193	57.28	0.9496	58	60	<0.0001
	2	100	39.59	1.55986	41	61	
	3	100	40.92	1.62858	40	72	
	4	80	50.61	1.96323	55	61	
	5	99	43.77	1.52987	46	60	
UAS-GCLC/+	1	163	38.55	1.32379	42	53	<0.0001
	2	97	35.71	1.40843	36	48	
	3	100	31.62	1.30823	31	52	
	4	97	34.47	1.38948	33	55	
	5	91	33.49	1.4498	31	51	

Although there is a statistically significant difference between the lifespan of driver control flies, responder control flies and D42 driven flies in experiments using male flies when GCLC is over-expressed in the motor neurons of the fly throughout development and adulthood, (Figure 4.5), when this difference is examined in detail (Table 4.3 and Table 4.4), it is evident that despite isogenisation, the genetic background of the driver and responder lines has a strong effect on longevity. When the *D42-GAL4* driven *UAS-GCLC* genotype (*UAS-GCLC/; D42-GAL4/+*) is compared to the heterozygous *UAS-GCLC* control, a statistically significant increase in longevity is seen in driven flies reflected by a 27.65% increase in mean lifespan, a 35.29% increase in median lifespan and a 30.19% increase in maximum lifespan (the heterozygous *UAS-GCLC* control has a mean lifespan of 35.22 days, a median of 34 days and a maximum of 53 days in comparison to 44.96 days, 46 days and 69 days respectively in the driven flies). Taken in isolation, this would appear to support the conclusion that over-expression of GCLC in the motor neurons alone does lead to a beneficial effect on longevity. However, *UAS-GCLC/+; D42-GAL4/+* flies, in which GCLC expression is driven in motor-neurons, show reduced mean (6.44%) and median (6.12%) compared to the *D42-GAL4* control flies. The groups are highly statistically significantly different and this is reflected by a 6.44% reduction in mean lifespan and a 6.12% reduction in median lifespan. Interestingly, the *UAS-GCLC/+; D42-GAL4/+* flies show a 2.99% increase in maximum lifespan compared to the heterozygous *D42-GAL4* control line, which has a mean lifespan of 48.06 days, a median of 49 days and a maximum of 67 days in comparison to 44.96 days, 46 days and 69 days respectively for the driven flies.

The source of the differential survival in these lines appears to be variations in their survival in the early, age-independent phase of the mortality curve (Phase I as described in Section 2.3.5 Methods and Materials). The heterozygous UAS-GCLC control has already reached 90% survival by approximately day 16 of the assay and the *D42-GAL4* driven *UAS-GCLC* line by approximately day 21, whilst the heterozygous *D42-GAL4* reaches 90% survival at approximately day 29 of the assay. The slopes of the steepest portion of the mortality curve (Phase II as described in Section 2.3.5 Methods and Materials), the portion where mortality is accelerating and where it becomes age-dependent and therefore the portion taken to represent the rate of ageing itself, is very similar between the three genotypes, as is Phase III of the curve (as described in Section 2.3.5 Methods and Materials).

The modest increase in maximum lifespan (2.99%) and the decrease in mean (6.44%) and median (6.12%) when the *D42-GAL4>GCLC* flies are compared to the driver control could imply that two separate effects are in place here. The presence of some element in the genetic background that causes non-senescent mortality in Phase I of the curve would lead to a reduction in both mean and median values as fewer individuals would survive the first two phases of the curve. It has been suggested that a maximum longevity increase is the strongest indicator of a physiological state providing protection from age-related conditions (Doubal and Klemera 1999). If this is the case, the small increase in maximum lifespan could be indicative of a protective effect despite the over-riding negative effect of the Phase I mortality. This will be discussed further in relation to other published work in Section 4.4. Nonetheless, based on these data, it is impossible to discern whether over-expression of GCLC in the motor neurons is truly advantageous to longevity. The increase

in early survival in lines containing a *D42-GAL4* chromosome masks any subtle effects that may occur in the age-dependent part of the mortality curve, making it impossible to disentangle the effects of variations in genetic background from those of the over-expression of the gene of interest. It is these variations in genetic background that represent the defining factor in the longevity of these strains, rather than driving UAS-GCLC in the motor neurons.

Figure 4.5 Lifespan assay for male flies from *D42-GAL4* driven *UAS-GCLC* lines and relevant controls. Each dataset is pooled data from 5-6 replicate tubs run simultaneously. The curves show a statistically significant difference (Log Rank test, p=<0.0001). Pairwise analysis of curves is detailed in Table 4.4.

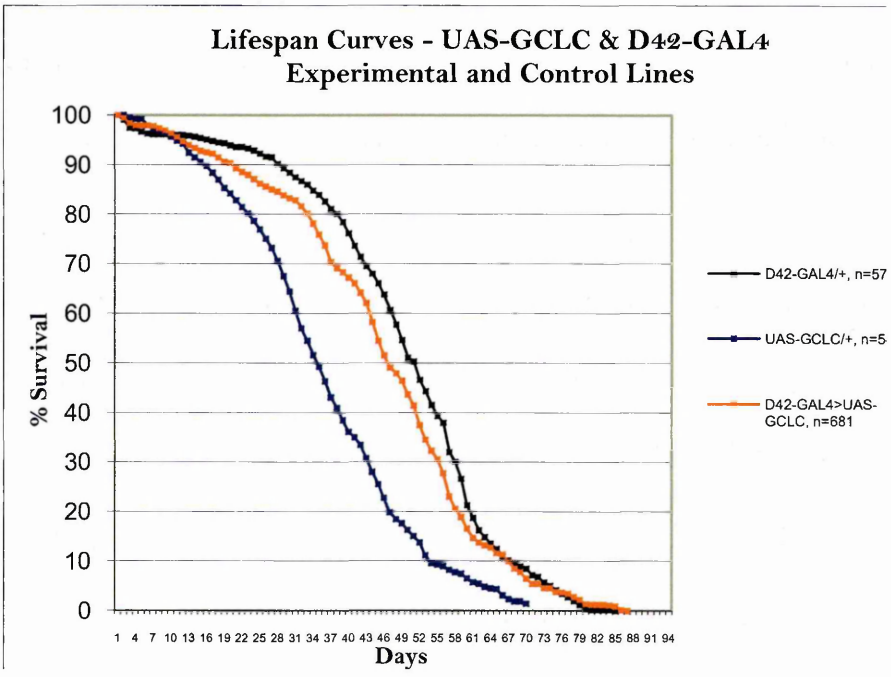


Table 4.3 Summary statistics for lifespan assay for male flies from *D42-GAL4* driver and *UAS-GCLC* responder experimental and control lines

Genotype	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p-value	DF
D42-GAL4/+	572	48.06	0.70395	49	67	<0.0001	2
UAS-GCLC/+	548	35.22	0.63358	34	53		
D42-GAL4>UAS-GCLC	681	44.96	0.66505	46	69		

Table 4.4 Pairwise comparison between genotypes for lifespan for male flies. In the case of both comparisons involving *D42-GAL4* driven *UAS-GCLC* flies, the figures refer to an increase or decrease in longevity of the driven flies relative to the control flies. In the case of the comparison of driver and responder control lines, the figures refer to an increase or decrease in longevity of the responder control flies relative to the driver control flies.

Comparison	% Change (Mean)	% Change (Median)	% Change (Max 90%)	Log Rank p-value	DF
D42-GAL4/+ vs D42-GAL4>UAS-GCLC	↓ 6.44	↓ 6.12	↑ 2.99	<0.0111	1
UAS-GCLC/+ vs D42-GAL4>UAS-GCLC	↑ 7.65	↑ 35.29	↑ 30.19	<0.0001	1
D42-GAL4/+ vs UAS-GCLC/+	↓ 26.71	↓ 30.61	↓ 20.90	<0.0001	1

Female Flies

To rule out any sex-specific effects, the *D42-GAL4* and *UAS-GCLC* lifespan assay was repeated using female flies. Individual replicates of each genotype were analysed to ascertain the amount of spread within each set of replicate tubs (Figure 4.6). Both the *D42-GAL4>UAS-GCLC* flies and the heterozygous *UAS-GCLC* control flies both showed a significant difference between individual replicates whereas the *D42-GAL4* heterozygous control did not (Table 4.5). However, taking into account uniformity of experimental conditions, these data were pooled and treated as a single dataset for the purposes of this analysis.

Figure 4.6 Individual replicates for lifespan assay for female flies from D42-GAL4 driver and UAS-GCLC responder line. Each replicate consists of an individual tub.

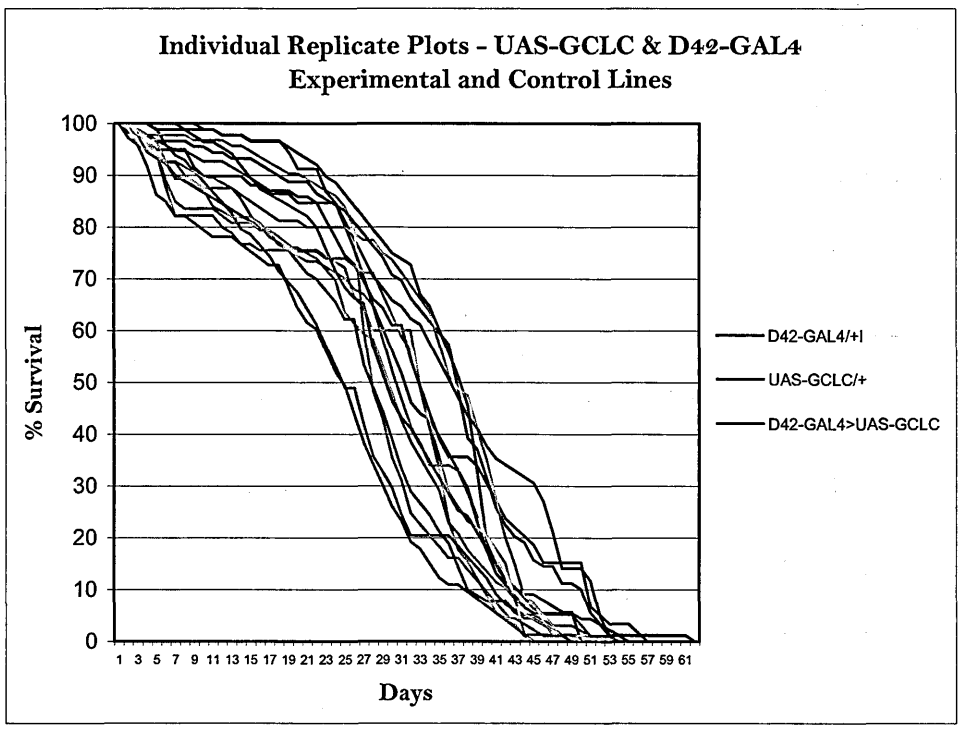


Table 4.5 Summary statistics for individual replicates from lifespan assay for female flies from D42-GAL4 driver and UAS-GCLC responder lines (experimental and control genotypes)

Genotype	Replicate	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value
D42-GAL4>UAS-GCLC	1	90	32.07	1.32985	37	44	<0.0001
	2	94	33.01	1.29705	35	47	
	3	93	33.23	0.81365	32	44	
	4	73	33.42	1.50214	37	47	
	5	80	37.06	1.42295	41	48	
D42-GAL4/+	1	85	39.75	1.36789	41	56	0.0382
	2	91	36.78	0.95998	36	47	
	3	88	39.34	0.913	42	48	
	4	59	37.02	1.74004	37	55	
	5	89	39.15	1.22636	41	55	
UAS-GCLC/+	1	90	30.47	1.20384	32	42	<0.0001
	2	73	27.19	1.35663	29	42	
	3	93	28.15	1.05538	29	45	
	4	95	34.04	1.16979	34	48	
	5	96	32.20	1.20658	34	47	

When GCLC is over-expressed in the motor neurons of female flies, there is a significant difference between driven and control groups (p=<0.0001) (Figure 4.7). However, as with male flies, this difference is likely to be the result of background genetic variation rather than the over-expression of GCLC. When the *D42-GAL4* driven *UAS-GCLC* line is compared to the heterozygous *UAS-GCLC* control, a statistically significant increase in longevity is seen in the driven flies reflected by a 10.14% increase in mean lifespan, a 12.5% increase in median lifespan and a 4.4% increase in maximum lifespan (the heterozygous *UAS-GCLC* control has a mean lifespan of 30.58 days, a median of 32 days and a maximum of 45 days compared with 33.68 days, 36 days and 47 days respectively for the driven line). As with male flies, taken in isolation this would appear to support the conclusion that over-expression of GCLC in the motor neurons alone leads to an increase in longevity. However, as with the male flies, it is the *D42-GAL4* heterozygous control that out-performs both lines with the *D42-GAL4* driven *UAS-GCLC* line showing a statistically significant impairment of longevity compared to the *D42-GAL4* heterozygous control line, a 12.50% reduction in mean lifespan, a 10% reduction in median lifespan and a 9.62% reduction in maximum lifespan (the heterozygous *D42-GAL4* control has a mean lifespan of 38.49 days, a median of 40 days and a maximum of 52 days in comparison to 33.68 days, 36 days and 47 days respectively for the driven line).

As with male flies, there is a variation in Phase I of the survival curve and it is far more pronounced in female flies: the *UAS-GCLC* heterozygous control line and the *D42-GAL4* driven *UAS-GCLC* line reach 90% survival at 7 and 8 days respectively in comparison to 21 for the *D42-GAL4* heterozygous control line. It is the presence or absence of the *D42-*

GAL4 chromosome that defines longevity in these lines but this appears to be independent of the effects of *D42-GAL4* driving over-expression of *UAS-GCLC* and a result of some other component of the genetic background of these flies, unrelated to the gene of interest.

Figure 4.7 Lifespan assay for female flies from *D42-GAL4* driven *UAS-GCLC* lines and relevant controls. Each dataset is pooled data from 5 replicate tubs run simultaneously. The curves show a statistically significant difference (Log Rank test, p=<0.0001). Pairwise analysis of curves is detailed in Table 4.7.

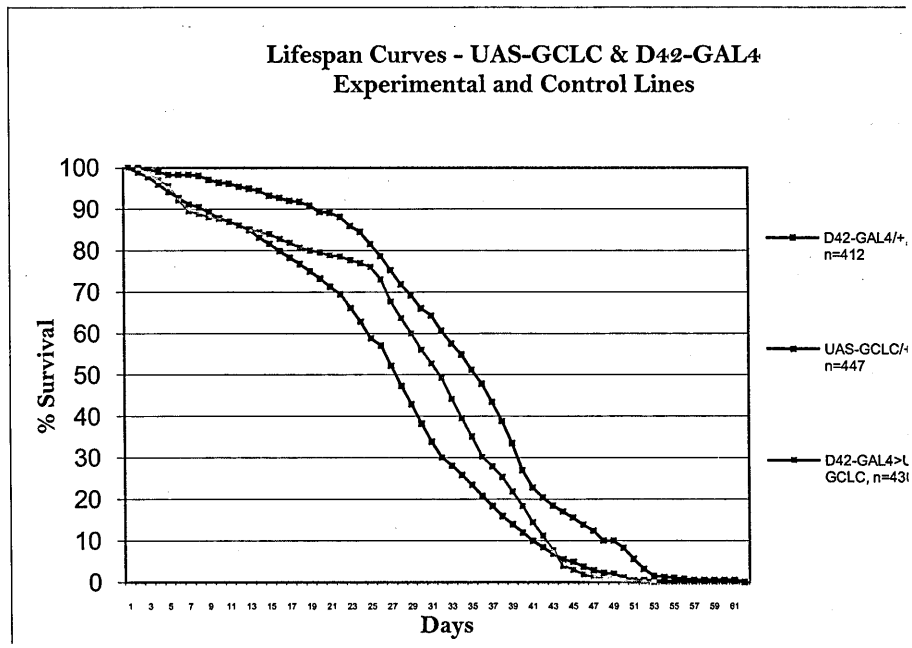


Table 4.6 Summary statistics for lifespan assay for female flies from *D42-GAL4* driver and *UAS-GCLC* responder experimental and control lines

Genotype	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p-value	DF
D42-GAL4/+	412	38.49	0.54373	40	52	<0.0001	2
UAS-GCLC/+	447	30.58	0.54441	32	45		
D42-GAL4>UAS-GCLC	430	33.68	0.57225	36	47		

Table 4.7 Pairwise comparison between genotypes for lifespan for female flies. In the case of both comparisons involving *D42-GAL4* driven *UAS-GCLC* flies, the figures refer to an increase or decrease in longevity of the driven flies relative to the control flies. In the case of the comparison of driver and responder control lines, the figures refer to an increase or decrease in longevity of the responder control flies relative to the driver control flies.

Comparison	% Change (Mean)	% Change (Median)	% Change (Max 90%)	Log Rank p-value	DF
D42-GAL4/+ vs D42-GAL4>UAS-GCLC	↓ 12.50	↓ 10	↓ 9.62	<0.0001	1
UAS-GCLC/+ vs D42-GAL4>UAS-GCLC	↑ 10.14	↑ 12.5	↑ 4.44	<0.0001	1
D42-GAL4/+ vs UAS-GCLC/+	↓ 20.74	↓ 20	↓ 13.46	<0.0001	1

4.3.2 Overexpression of GCLC and GCLM in motor neurons

It is possible that the confounding background effects seen in the *UAS-GCLC D42-GAL4* experiments were specific to the *UAS-GCLC* strain and that the *UAS-GCLC,GCLM* holoenzyme mimic recombinant strain could still show expression-specific differences in longevity, independent of background variation. Although the source of the *UAS-GCLC* element in this strain is the strain used in the previous section, the recombinant strain also has a *UAS-GCLM* element present and underwent recombination in the creation of the strain which would have resulted in a unique and separate genetic background to the *UAS-GCLC* strain. The recombinant strain provides a more balanced over-expression situation as it is unlikely that, as might be the case when GCLC is over-expressed in isolation, that lower endogenous levels of GCLM will be the limiting factor in the lifespan assay.

Male Flies

When individual replicates were analysed, there was a significant difference between replicates for both control genotypes and the experimental *D42-GAL4* driven *UAS-GCLC,GCLM* lines (Figure 4.8, Table 4.8). However, taking into account uniformity of experimental conditions, these data were pooled and treated as a single dataset for the purposes of this analysis.

Figure 4.8 Individual replicates for lifespan assay for male flies from *D42-GAL4* driver and *UAS-GCLC, GCLM* responder line. Each replicate consists of an individual tub.

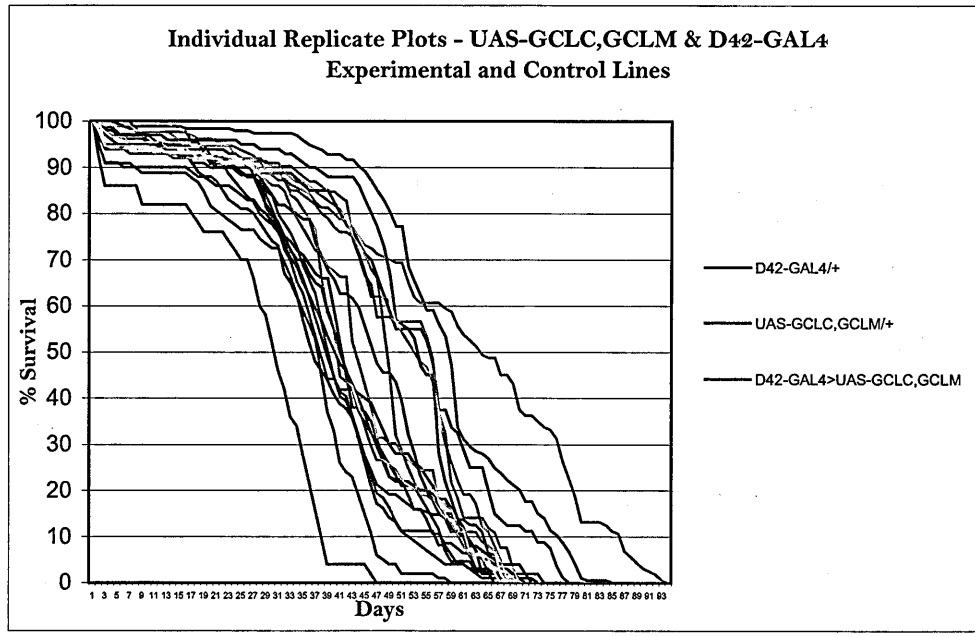


Table 4.8 Summary statistics for individual replicates from lifespan assay for male flies from *D42-GAL4* driver and *UAS-GCLC,GCLM* responder lines (experimental and control genotypes)

Genotype	Replicate	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value
D42-GAL4>UAS-GCLC,GCLM	1	92	48.63	1.66329	53	66	<0.0001
	2	94	41.85	1.33995	40	61	
	3	100	44.95	1.50221	48	63	
	4	99	48.59	1.63816	55	65	
	5	86	42.47	1.57665	43	60	
D42-GAL4/+	1	100	39.59	1.55986	41	60	<0.0001
	2	100	40.92	1.62858	40	61	
	3	80	50.61	1.96323	55	72	
	4	99	43.77	1.52987	46	61	
	5	100	50.1	1.24839	53	60	
UAS-GCLC,GCLM/+	1	94	38.63	1.40301	38	57	<0.0001
	2	43	39.23	1.98662	37	57	
	3	50	26.12	1.77618	30	37	
	4	100	33.19	1.30776	37	46	
	5	99	37.44	1.34505	39	52	
	6	98	34.86	1.61969	36	56	

As was the case with over-expression of GCLC in the motor neurons, motor neuron expression of both GCLC and GCLM leads to significant lifespan extension relative to only one of the two control lines (Figure 4.9). When lifespan curves for *D42-GAL4>UAS-GCLC,GCLM* males are compared with the heterozygous *UAS-GCLC,GCLM* control flies, there is a highly significant difference between the curves. The *D42-GAL4>UAS-GCLC,GCLM* flies have a 24.90% higher mean lifespan, a 24.32% higher median lifespan and 25.45% higher maximum lifespan (the heterozygous *UAS-GCLC,GCLM* control line has a mean lifespan of 35.26 days, a median of 37 days and a maximum of 55 days compared to 44.04 days, 46 days and 69 days respectively for the *D42-GAL4* driven *UAS-GCLC,GCLM* line). However, when *D42-GAL4>UAS-GCLC,GCLM* flies are compared to the *D42-GAL4* heterozygous control flies, there is no statistically significant difference in the survival curves. As with the catalytic subunit alone, the driven flies showed a minor decrease in mean lifespan (1.63%) compared to the heterozygous driver control, a minor decrease in median lifespan (2.13%) and a small increase in maximum lifespan 2.99% but the groups are not statistically significantly different (heterozygous *D42-GAL4* control flies have a mean lifespan of 44.77 days, a median of 47 days and a maximum of 67 days in comparison to 44.04 days, 46 days and 69 days respectively for the driven flies). It is, therefore, impossible to conclude that the observed increase in longevity is necessarily due to the expression of GCL in motor neurons as, whilst there may be an effect relative to the responder control, it is not a large enough effect to extend lifespan past the second control line. Thus, it appears once again that the presence of the driver chromosome is the defining factor in the longevity of these lines.

Figure 4.9 Lifespan assay for male flies from *D42-GAL4* driven *UAS-GCLC,GCLM* lines and relevant controls. Each dataset is pooled data from 5-6 replicate tubs run simultaneously. The curves show a statistically significant difference (Log Rank test, p=<0.0001). Pairwise analysis of curves is detailed in Table 4.9.

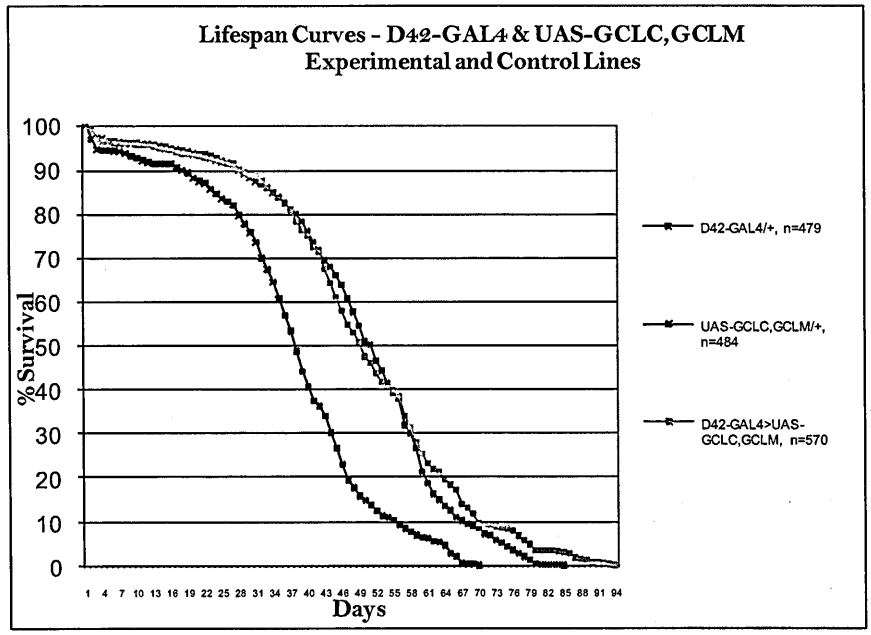


Table 4.9 Summary statistics for lifespan assay for male flies from *D42-GAL4* driver and *UAS-GCLC,GCLM* responder experimental and control lines

Genotype	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p-value	DF
D42-GAL4/+	479	44.77	0.73198	47	67	<0.0001	2
UAS-GCLC,GCLM/+	484	35.26	0.64854	37	55		
D42-GAL4>UAS-GCLC,GCLM	570	44.04	0.65686	46	69		

Table 4.10 Pairwise comparison between genotypes for lifespan for male flies. In the case of both comparisons involving *D42-GAL4* driven *UAS-GCLC,GCLM* flies, the figures refer to an increase or decrease in longevity of the driven flies relative to the control flies. In the case of the comparison of driver and responder control lines, the figures refer to an increase or decrease in longevity of the responder control flies relative to the driver control flies.

Comparison	% Change (Mean)	% Change (Median)	% Change (Max 90%)	Log Rank p-value	DF
D42-GAL4/+ vs D42-GAL4>UAS-GCLC,GCLM	↓ 1.63	↓ 2.13	↑ 2.99	0.1271	1
UAS-GCLC,GCLM/+ vs D42-GAL4>UAS-GCLC,GCLM	↑ 24.90	↑ 24.32	↑ 25.45	<0.0001	1
D42-GAL4/+ vs UAS-GCLC,GCLM/+	↓ 21.24	↓ 21.28	↓ 17.91	<0.0001	1

Female Flies

The *D42-GAL4* and *UAS-GCLC,GCLM* lifespan assay was repeated using female flies. Individual replicates of each genotype were analysed to ascertain the amount of spread within each set of replicate tubs (Figure 4.10). Although two out of the three lines did show a significant difference (Table 4.11), these data were pooled and treated as a single dataset for the purpose of this analysis as experimental conditions were uniform.

Figure 4.10 Individual replicates for lifespan assay for female flies from *D42-GAL4* driver and *UAS-GCLC, GCLM* responder line. Each replicate consists of an individual tub.

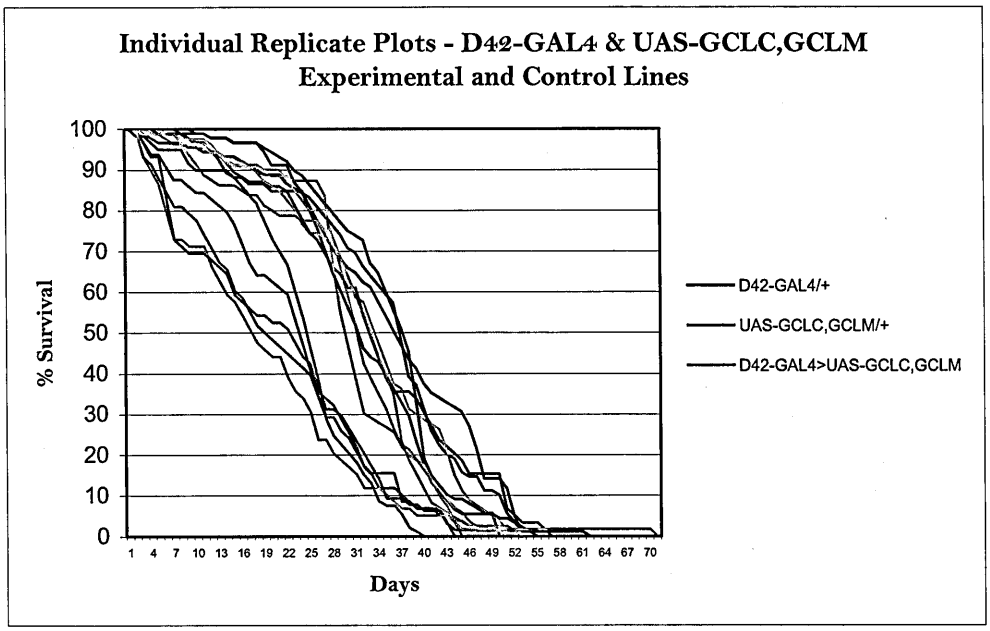


Table 4.11 Summary statistics for individual replicates from lifespan assay for female flies from *D42-GAL4* driver and *UAS-GCLC,GCLM* responder lines (experimental and control genotypes)

Genotype	Replicate	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value
D42-GAL4>UAS-GCLC,GCLM	1	86	35.37	1.01615	38	45	<0.0001
	2	80	35.58	1.01489	36	46	
	3	86	35.00	0.97226	34	47	
	4	80	36.28	1.37898	38	50	
	5	95	40.69	1.2406	41	55	
D42-GAL4/+	1	85	39.75	1.36789	41	56	0.0382
	2	91	36.78	0.95998	36	47	
	3	88	39.34	0.913	42	48	
	4	59	37.02	1.74004	37	55	
	5	89	39.15	1.22636	41	55	
UAS-GCLC,GCLM/+	1	59	24.83	1.61038	27	42	0.7353
	2	64	27.33	1.41124	28	39	
	3	58	24.88	1.59477	24	41	
	4	93	28.63	0.7555	29	38	
	5	59	23.39	1.71066	22	40	

Figure 4.11 shows survival curves for this experiment and there is a highly statistically significant difference between these groups (Table 4.12). However, as with previous experiments, the defining factor in this difference seems to be a background effect unrelated to the over-expression of both GCL sub-units. Pairwise analysis of genotypes shows that, whilst the driven flies show a highly statistically significant difference when compared to the responder control reflected in a 40.41% increase in mean lifespan, a 37.04% increase in median lifespan and a 21.95% increase in maximum lifespan, these driven flies show a highly statistically significant impairment relative to the driver control reflected in a 4.68% decrease in mean lifespan, a 7.5% decrease in median lifespan and a 3.85% decrease in maximum lifespan. Therefore, as with male flies, it appears to be the presence of the driver chromosome that defines longevity in these flies rather than the expression-status of the GCL subunits.

Figure 4.11 Lifespan assay for female flies from *D42-GAL4* driven *UAS-GCLC,GCLM* lines and relevant controls. Each dataset is pooled data from 5 replicates run simultaneously. The curves show a statistically significant difference (Log Rank, p=<0.0001). Pairwise analysis of curves is detailed in Table 4.13

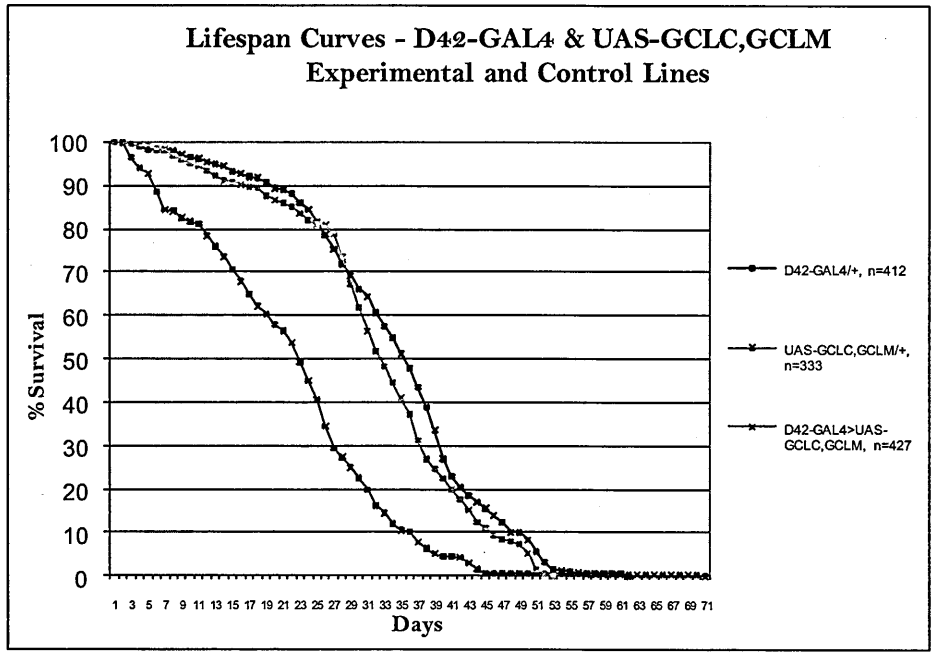


Table 4.12 Summary statistics for lifespan assay for female flies from *D42-GAL4* driver and *UAS-GCLC,GCLM* responder experimental and control lines

Genotype	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p-value	DF
D42-GAL4/+	412	38.49	0.54373	40	52	<0.0001	2
UAS-GCLC,GCLM/+	333	26.13	0.61282	27	41		
D42-GAL4>UAS-GCLC,GCLM	427	36.69	0.51775	37	50		

Table 4.13 Pairwise comparison between genotypes for lifespan for female flies. In the case of both comparisons involving *D42-GAL4* driven *UAS-GCLC,GCLM* flies, the figures refer to an increase or decrease in longevity of the driven flies relative to the control flies. In the case of the comparison of driver and responder control lines, the figures refer to an increase or decrease in longevity of the responder control flies relative to the driver control flies.

Comparison	% Change (Mean)	% Change (Median)	% Change (Max 90%)	Log Rank p-value	DF
D42-GAL4/+ vs D42-GAL4>UAS-GCLC,GCLM	↓ 4.68	↓ 7.5	↓ 3.85	0.0020	1
UAS-GCLC,GCLM/+ vs D42-GAL4>UAS-GCLC,GCLM	↑ 40.41	↑ 37.04	↑ 21.95	<0.0001	1
D42-GAL4/+ vs UAS-GCLC,GCLM/+	↓ 32.11	↓ 32.5	↓ 21.15	<0.0001	1

4.4. Discussion

Evidence in support of the role of motor neurons as a lifespan limiting tissue-type has been published from over-expression studies in *Drosophila* involving both GCL and SOD1 (Orr et al 2005; Parkes et al; 1998; Parkes et al 1999; Luchak et al 2007). Work by Orr and colleagues, over-expressing individual GCL subunits (Orr et al 2005) is particularly relevant to the work presented in this thesis.

The lifespan assay protocol described in this thesis differs significantly from that of Orr *et al* (2005). Orr and colleagues housed their flies in vials of 20 flies and these were transferred to fresh media on a daily basis. The results from all vials were then pooled and treated as a single replicate experiment. There are several drawbacks to this approach. In lifespan studies, it is necessary to minimise any environmental variables which could confound the analysis by affecting the flies. Each vial, in essence, comprises an individual micro-environment and flies in that vial could be exposed to different stresses, for example a bacterial infection in the media. In order to minimise the risk of this kind of variation, the protocol adopted in our laboratory used larger tubs to enable more flies to be comfortably housed in a single micro-environment (an individual tub). In addition, daily passage of flies from vial to vial is more invasive than simply switching out a vial at the top of a tub and also leads to a higher loss rate of flies within each experiment as a result of escapee flies or flies getting caught in the bungs of the vials. Whilst there was still a small number of escapee flies from the tubs used here, they comprised a much smaller percentage of the total number of flies in each micro-environment. In addition, flies are able to fly within the tubs for short distances and have been observed to do so. This provides a closer approximation

of activity levels for free flying animals, although still at a reduced level, than vials where there is no room for flight. For these reasons, it was decided to adopt the protocol described in this thesis rather than that of housing flies in smaller vials. Nonetheless, these differences must be taken into account when comparing the results between laboratories.

4.4.1 GCLM

When GCLM was over-expressed specifically in the motor neurons of male flies, no significant lifespan extension was seen despite significant extension when it was over-expressed globally (Orr et al 2005). This does not support the hypothesis that motor neurons are the key lifespan limiting tissue as ubiquitous over-expression in all tissue types including the motor neurons gives significant lifespan extension but specific over-expression in the motor neurons alone does not. There could be various explanations for this result. It is possible that lifespan extension due to GCLM over-expression in motor-neurons is level-dependent and ubiquitous over-expression using a high level driver raises expression levels in motor neurons above a threshold that gives rise to lifespan extension. The *D42-GAL4* driver may not drive expression at these levels. It is also, however, possible that the significant extension seen when GCLM is over-expressed ubiquitously was a background effect, specific to the strains used by Orr and colleagues. This suggestion is supported by the fact that, as detailed in Chapter 3 of this thesis, this result was not replicated by work presented here.

4.4.2 GCLC and the Holoenzyme Mimic

The relationship between GCLC over-expression in motor neurons and lifespan extension in work presented by Orr and colleagues is strong and replicable (see Table 4.14 for a summary of mean values) (Orr et al 2005).

Table 4.14 Comparison of mean lifespan data from over-expression studies using male flies involving *UAS-GCLC* and *D42-GAL4*. All values cited are mean values. Data in the second column is taken from work published by Orr and colleagues and comprises 3 individual insertion lines of the UAS-GCLC transgene (Orr et al 2005). Data in the third column is discussed in Section 4.2.1 of this thesis.

Genotype	Mean (Days)	Mean (Days)
GCLC/+	54.2	35.22
	53.3	
	55.9	
D42/+	54.8	48.06
D42>GCLC	72.2	44.96
	73.1	
	74.9	

Orr and colleagues reported greater than 30% mean lifespan extension relative to either control line whereas the results presented in this thesis show greater than 30% extension only relative to the responder control line, with the driver control line out-performing the driven flies in mean and median lifespan but showing a small reduction in maximum lifespan. When mean values are compared between this thesis and the work by Orr and colleagues (Table 4.14), it is evident that a severely reduced mean lifespan in the *UAS-GCLC* responder control relative to both the driven flies and the driver control is responsible for the greater than 30% extension discussed in Section 4.3.1. Overall, our fly strains show reduced mean lifespan values relative to the published values (Table 4.14) (as discussed in Section 3.4, this could be a result of strain-specific lifespan differences between laboratories or differences in the lifespan assay protocol itself). It is possible that the factor

that causes death in the early phases of the lifespan curves of our *UAS-GCLC* lines is exerting such a strong effect that it overwhelms the positive effect on lifespan of over-expression in motor neurons and that, had this been effectively removed by isogenisation crosses, we may have seen extension similar to that seen by Orr and colleagues. There is a small increase in maximum lifespan which could be indicative of this potential, however, this is purely speculation. Work by Parkes and colleagues over-expressing human SOD1, another component of the antioxidant defence system which has been suggested to work in tandem with glutathione to maintain the oxidative balance in organisms, in motor neurons (Winterbourn 1993), also shows significant increases in mean and maximum lifespan relative to the responder control (Parkes et al 1998). Whilst the actual data are not shown for the heterozygous D42-GAL4 driver control line in this paper, they are described as being similar to the heterozygous responder control line and, therefore, not a defining factor in the extension shown.

This background effect resulting in impaired responder control mean and median lifespan and potentially reduced driven responder mean and median lifespan (evident from a reduction in both mean and median relative to the heterozygous driver control) also appears to be dominant in female flies over-expressing GCLC in the motor neurons (Section 4.3.1) and in both sexes over-expressing both the holoenzyme subunits (Section 4.3.2). Therefore, despite the published evidence in support of the hypothesis that motor neurons are a lifespan-limiting tissue in *Drosophila*, this thesis has been unable to present data that support this evidence. What is clear is that the genetic background of strains used in lifespan assays has a strong influence on the results despite isogenisation crosses. This

raises the question of whether fly strains can ever be truly isogenised by backcrossing alone. Certainly with regards to this particular *D42-GAL4* driver, it appears that there must be some variation quite closely linked to the P-element insertion which is associated with longevity. This is a limitation when a back-crossing strategy is employed to isogenise lines as elements that are closer together are far less likely to be subject to recombination events than those separated by a greater distance. It is necessary, therefore, in lifespan assays for an expression system to be used that bypasses the issue of genetic background completely. Results presented in Chapter 5 of this thesis use such a system, the Geneswitch system (described in Section 1.7.3), to further investigate the role of neural tissue deterioration in ageing.

5. EFFECT OF OVER-EXPRESSION OF GCL IN A PAN-NEURAL PATTERN ON LIFESPAN AND STRESS RESISTANCE IN *DROSOPHILA MELANOGASTER*

5.1. Introduction

The data presented and discussed in Chapters 3 and 4 demonstrate that both genetic background and developmental effects can strongly influence the lifespan characteristics of a particular fly strain. Whilst work by Orr *et al* (2003) suggests a key role for the nervous system of the fly in influencing longevity, this has not been unequivocally shown to be the case for over-expression of GCL in motorneurons (Chapter 4). Nonetheless, it is still possible that over-expression of GCL in neural tissue could affect lifespan. Reductions in the level of SOD2 in *Drosophila* lead to neurodegeneration and reduce lifespan and age-dependent mortality (Paul, Belton et al. 2007) implying that neural tissue is sensitive to manipulation of the oxidative defence enzymes in *Drosophila.* However, as discussed in Section 1.6.2, a reduction in lifespan due to any intervention that deviates from the biological norm for a specific tissue, does not necessarily imply a causative role in ageing. Nonetheless, it does suggest that neural tissue is sensitive to a reduction in organismal antioxidant defence enzymes. If this is the case, it is possible that neural tissue is particularly sensitive to oxidative damage and hence that over-expression of GCL subunits and the predicted consequent increase in glutathione levels could provide individuals with increased protection from this damage hence reducing mortality.

The use of the *D42 Gal4* driver to investigate the consequences of GCL expression in the nervous system proved problematic, as described in the preceding chapter. The Geneswitch system (described in Section 1.7.3) provides the most effective means of reducing the impact of confounding variables (most notably that due to genetic background of the driver and responder strains), by enabling conditional expression of the gene of interest in *genetically identical* fly genotypes. This is made possible because Geneswitch constructs express a Gal4-progesterone receptor that can only drive expression from the Gal4 UAS when bound to the synthetic progesterone hormone (RU486) delivered via the food media. This system also enables temporal control of expression, allowing the bypass of pre-adult expression thereby removing confounding developmental effects. This enables expression of the gene of interest solely during adult lifespan and hence isolates its effects to a post-developmental period.

For the reasons described previously (Section 3.1), the work described in this chapter seeks to evaluate the consequences of over-expression of GCLC and GCLM separately and in combination using the Geneswitch system. In addition, both male and female flies were assayed to examine whether any observed effects were sex specific possibly as a consequence of differential metabolic loads related to reproduction.

5.2. Fly Strains and Procedures

5.2.1 Fly Strains

The fly strains used in these experiments are detailed in Table 5.1 alongside the abbreviations that have been used in this Chapter.

Table 5.1 Fly strains referred to in this chapter (see Section 2.1 Methods & Materials for full details of each strain)

Fly Strain	Abbreviation	P-Element Chromosome	Description
w^{1118}; p{UAST GCLM T7.3.1 w^+}	UAS-GCLM	II	Containing *Drosophila* GCL transgenes in pUAST vector
w^{1118};p{UAST GCLC T2.1.3 w^+}	UAS-GCLC	II	
w^{1118};p{UAST GCLC T2.1.3 w^+}, p{UAST GCLM T7.3.1 w^+}	UAS-GCLC,GCLM	II	
y^1 w^*; ; P{elav-GS}	elav-GS	III	Pan-neural expression induced by RU486 ingestion

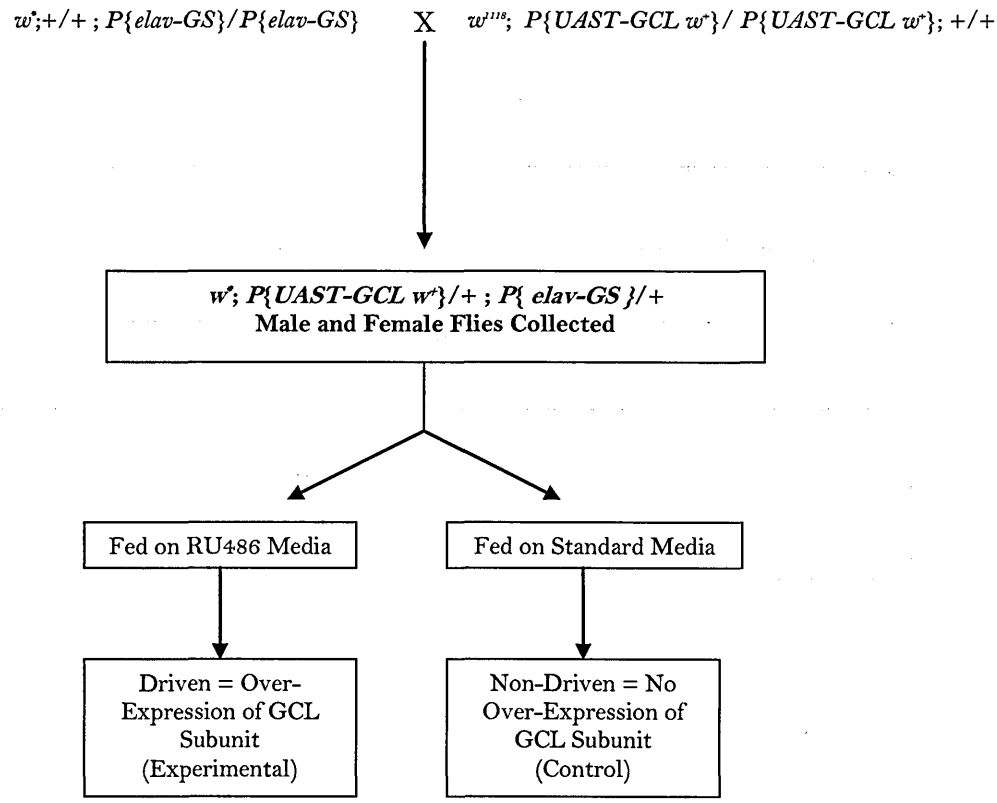
5.2.2 Procedures

Generation of Experimental Genotypes

In order to investigate whether over-expression of GCLM in neural tissue altered longevity, flies were generated using the crossing scheme in Figure 5.1. The elav-GS driver combines cloned promoter fragments of the *embryonic lethality abnormal vision* (*elav*) gene and the GAL4-progesterone receptor fusion protein, enabling the creation of a genetically homogenous line where expression in a pan-neural pattern is controlled by the presence or absence of the synthetic progesterone hormone, RU486 in food media (Figure 5.1). Expression was verified by crossing with a *UAS-GFP* stock (data not shown). This crossing scheme was carried out using *UAS-GCLM, UAS-GCLC* and *UAS-GCLC,GCLM* in order to assess the effects of all combinations of sub-unit over-expression. In all cases, over-

expression is in a wild-type background and is therefore expression above endogenous levels.

Figure 5.1 Crossing scheme used to generate flies for *elav-GS* lifespan.



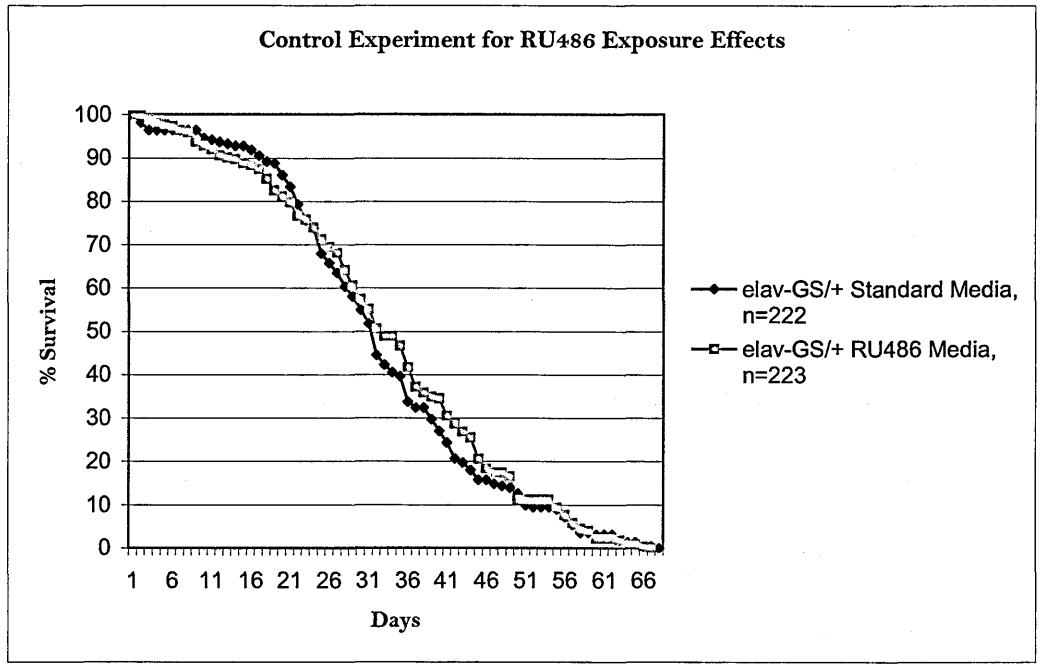
Lifespan Assays

Lifespan assays were carried out according to the protocol described in Section 2.3.2 Methods and Materials, with minor alterations related to administering RU486. On eclosion, male and female flies were transferred to fresh bottles of standard medium without anaesthesia where they were aged together for 48 hours. This reduced the likelihood of there being a mix of virgin and non-virgin flies in the female assays. In addition, this delayed exposure to RU486 in the food media to 48 hours post-eclosion, clearly separating induction of over-expression from any developmental or early post-eclosion effects which could complicate the analysis. Due to daily eclosion volume constraints, individual replicate tubs contained fewer flies than those described in Chapter 4. For full details of the feeding protocol, see Section 2.3.2 Materials and Methods.

The Effect of RU486 Exposure on *Drosophila melanogaster*

Previously, RU486 has been reported to have no effect on longevity in *Drosophila* (Osterwalder, Yoon et al. 2001; Poirier, Shane et al. 2008). In order to verify that in our hands, flies heterozygous for a chromosome containing elav-GS were exposed to RU486 and a lifespan assay was carried out according to the protocol in Section 2.3.2. The results shown below in Figure 5.2 confirm that RU486 has no effect on longevity.

Figure 5.2 Lifespan assay investigating the effects of RU486 on male *Drosophila melanogaster*. Flies of the genotype *w*; elav-GS/+* were generated by crossing the *elav-GS* driver line to the laboratory reference stock, w^{1118}. Each curve below comprises pooled data from 4 tubs per genotype. Tubs contained between 50 and 56 flies. The lifespan curves are not significantly different (p=0.4126, Log rank test)



Statistical Analyses

Statistical analyses were carried out as described in Section 2.3.3.

5.3. Results

5.3.1 Over-Expression of GCLM

Males

In the first instance, individual replicates were analysed to provide a statistical analysis of the spread of the replicate curves (Figure 5.3). While there was a statistically significant difference between the individual replicates (Table 5.2), as experimental conditions were

uniform and all the control and experimental replicates were fed on media from the same batch, replicates were pooled for the purpose of this analysis.

Figure 5.3 Individual replicates for lifespan assay for male *UAS-GCLM/+; elav-GS/+* flies (experimental and control treatments). Each replicate consists of an individual tub.

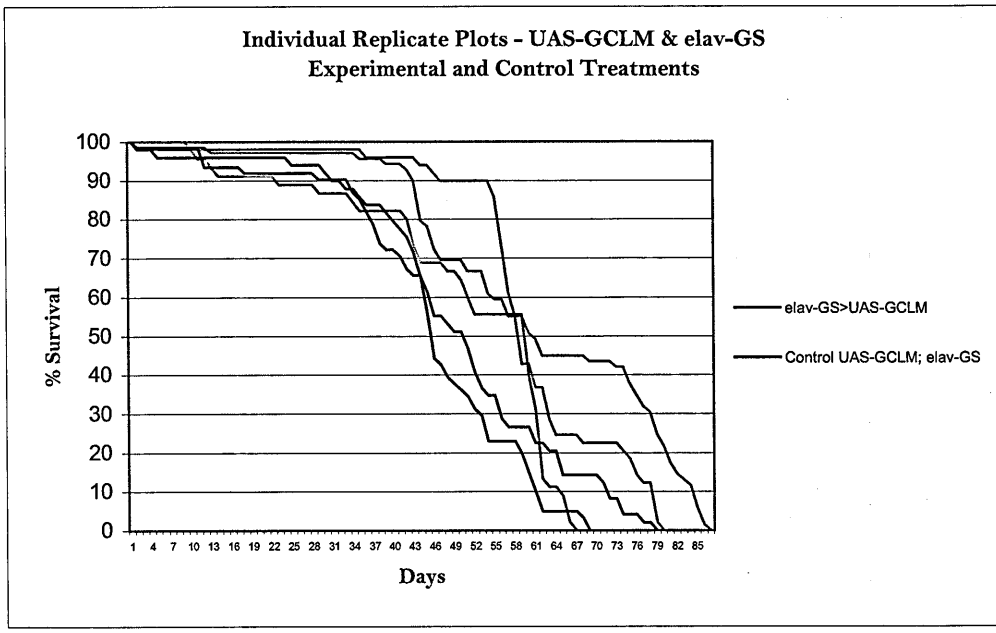


Table 5.2 Summary statistics for individual replicates from lifespan assay for male *UAS-GCLM/+; elav-GS/+* flies (experimental and control treatments).

Genotype	Treatment	Replicate	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value
UAS- GCLM/+; elav-GS/+	+RU486	1	49	59.71	1.88035	58	80	<0.0001
		2	69	61.39	2.25658	60	86	
		3	45	49.78	2.40934	59	66	
	-RU486	1	49	48.88	2.30731	50	73	0.0323
		2	61	44.67	1.80326	45	62	

When GCLM is over-expressed in a pan-neural pattern in adult males, a statistically significant increase in longevity is seen. This manifests as a 23.91% increase in mean lifespan, a 31.11% increase in median lifespan and an 22.73% increase in maximum lifespan (*elav-GS* driven *UAS-GCLM* flies have a mean lifespan of 57.68 days, a median of 59 and a maximum of 81 days in comparison to 46.55, 45 and 66 respectively for non-driven flies) (Figure 5.4 and Table 5.3).

In contrast to the data shown in Chapters 3 and 4, where over-expression was induced during early embryogenesis and persisted throughout larval and pupal development, these data do not show high age-independent mortality in Phase I of the lifespan curve. Over-expression of GCLM in neural tissue appears to delay the onset of ageing – the point of maximal slope change is at approximately 43 days for the RU486-treated flies in comparison to 34 days for control flies (see Section 2.3.5 for a discussion of how this point is determined). In addition, the slope of the Phase II part of the curve is shallower in places in RU486-treated flies, implying that over-expression of GCLM in neural tissue may lower the rate of ageing in these flies. The *elav-GS* driven *UAS-GCLM* flies also show an improvement in late-life survival (Phase III of the curve), implying that individuals in which GCLM is over-expressed in neural tissue and which survive past Phase II of the lifespan assay still retain a survival advantage over their control siblings.

Figure 5.4 Lifespan assay for male *UAS-GCLM/+; elav-GS/+* flies (experimental and control treatments). Each dataset is pooled data from 2-3 replicate tubs run simultaneously. The curves show a statistically significant difference (log rank test, p=<0.0001).

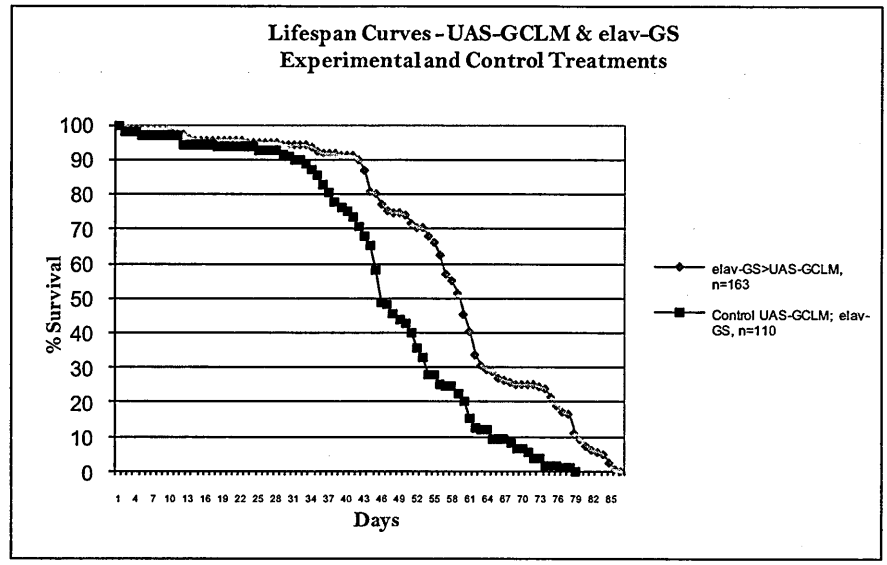


Table 5.3 Summary statistics for lifespan assay for male *UAS-GCLM/+; elav-GS/+* flies (experimental and control treatments). The percentage change figures for mean, median and maximum lifespan refer to the percentage change of driven flies relative to non-driven flies.

Genotype	Treatment	n (Flies)	Mean (days)	SEM	Median (days)	Max (90%) (days)	% Change Mean	% Change Median	% Change Max	Onset of Ageing (days)	Log Rank p value	DF
UAS- GCLM/+; elav-GS/+	+RU486	163	57.68	1.34354	59	81	↑ 23.91	↑ 31.11	↑ 22.73	43	<0.0001	1
	-RU486	110	46.55	1.44118	45	66				34		

Females

In order to investigate whether the lifespan extension seen following pan-neural expression of GCLM in males flies was a sex-specific effect, GCLM was over-expressed in neural tissue in female flies, using the same Geneswitch driver system. It is possible that the increased metabolic load on females from egg production and laying could lead to a different response to GCLM over-expression.

Initially individual replicates for driven and non-driven flies were assessed and found to be statistically significantly different (Figure 5.5 and Table 5.4). However, for the same reasons as for male flies, these replicates were pooled for the purpose of this analysis.

Figure 5.5 Individual replicates for lifespan assay for female *UAS-GCLM/+; elav-GS/+* flies (experimental and control treatments).

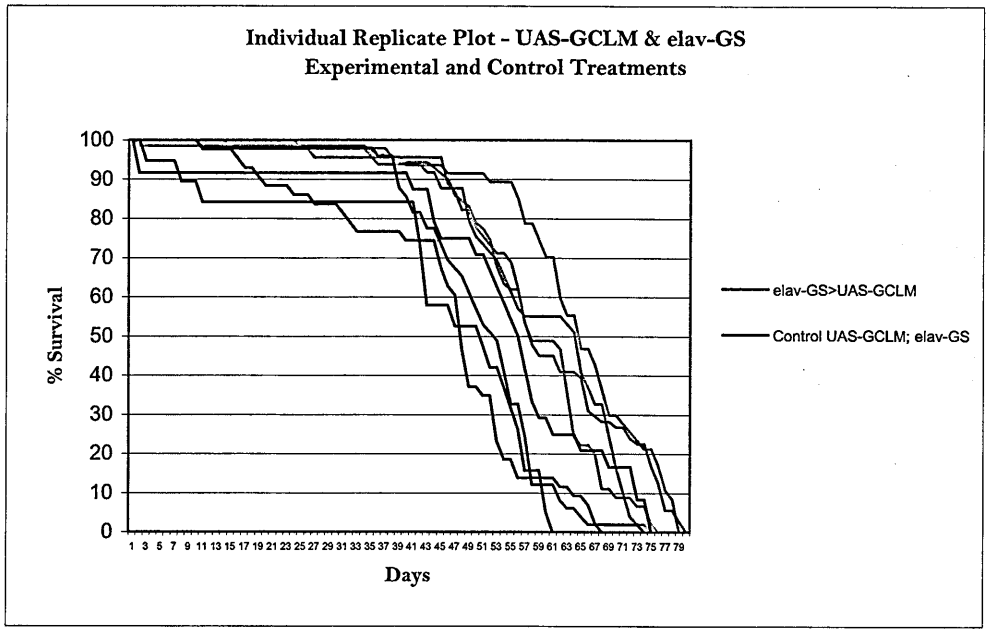


Table 5.4 Summary statistics for individual replicates from lifespan assay for *female UAS-GCLM/+; elav-GS/+* flies (experimental and control treatments).

Genotype	Treatment	Replicate	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value
UAS- GCLM/+; elav-GS/+	+RU486	1	47	63.47	1.6575	64	79	0.0065
		2	45	57.04	1.78488	57	71	
		3	71	59.07	1.61262	57	78	
		4	49	58.37	1.75199	64	73	
	-RU486	1	43	44.53	2.21251	47	65	0.0102
		2	24	52.25	3.79371	56	74	
		3	49	49.96	1.4413	52	63	
		4	19	43.47	4.08203	50	61	

As was seen with male flies, over-expression of GCLM in neural tissue leads to a statistically significant increase in longevity ($p=<0.0001$). Driven flies show a 24.55% increase in mean lifespan, a 22% increase in median lifespan and a 18.46% increase in maximum lifespan (*elav-GS* driven *UAS-GCLM* flies have a mean lifespan of 59.45 days, a median of 61 days and a maximum of 77 days in comparison to 47.73 days, 50 days and 65 days respectively for control flies) (Figure 5.6 and Table 5.5). Unlike male flies, whilst there is a small decrease in the rate of ageing in driven flies exemplified by a minor shallowing of Phase II of the lifespan curve, this is not the prime source of the deviation. Instead, female non-driven flies show early age-independent mortality in Phase I of the curve, which is not seen in driven flies. As with male flies, the onset of ageing is later in driven flies (47 days) than control flies (40 days). However, unlike male flies, driven flies show no reduced mortality rate late in Phase III of the lifespan curve. Nonetheless, the maximum lifespan of the experimental flies remains greater than that of control flies (77 days in driven flies in comparison to 65 for control flies).

Figure 5.6 Lifespan assay for female *UAS-GCLM/+; elav-GS/+* flies (experimental and control treatments). Each dataset is pooled data from 4 replicate tubs run simultaneously. The curves show a statistically significant difference (log rank test, p=<0.0001).

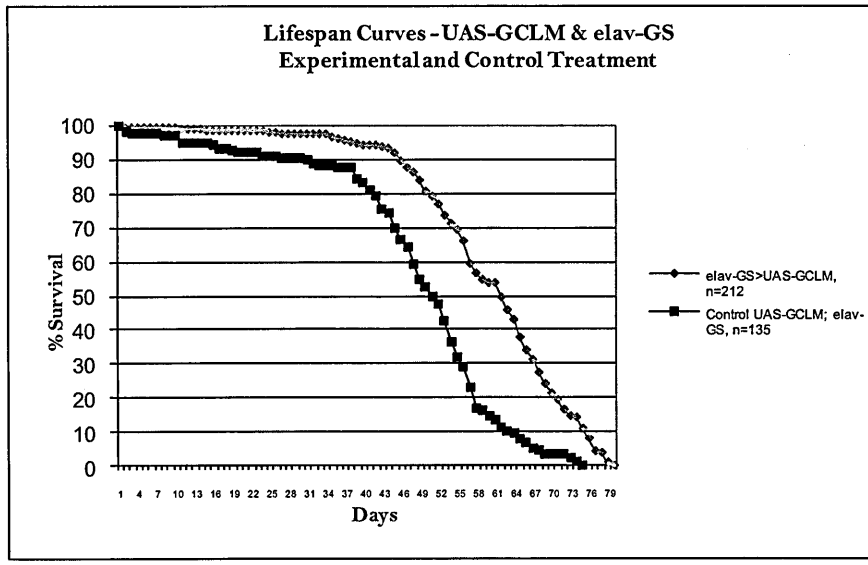


Table 5.5 Summary statistics for lifespan assay for female *UAS-GCLM/+; elav-GS/+* flies (experimental and control treatments). The percentage change figures for mean, median and maximum lifespan refer to the percentage change of driven flies relative to non-driven flies.

Genotype	Treatment	n (Flies)	Mean (days)	SEM	Median (days)	Max (90%) (days)	% Change Mean	% Change Median	% Change Max	Onset of Ageing (days)	Log Rank p value	DF
UAS- GCLM/+; elav-GS/+	+RU486	212	59.45	0.86512	61	77	↑ 24.55	↑ 22.00	↑ 18.46	47	<0.0001	1
	-RU486	135	47.73	1.26366	50	65				40		

5.3.2 Over-Expression of GCLC

Males

Initially, experimental and control replicates were assessed for spread and found to differ (Figure 5.7 and Table 5.6). These replicates were run simultaneously, under identical conditions, fed on media from the same batch and therefore were pooled for the purpose of this analysis.

Figure 5.7 Individual replicates for lifespan assay for male *UAS-GCLC/+; elav-GS/+* flies (experimental and control treatments). Each replicate consists of an individual tub.

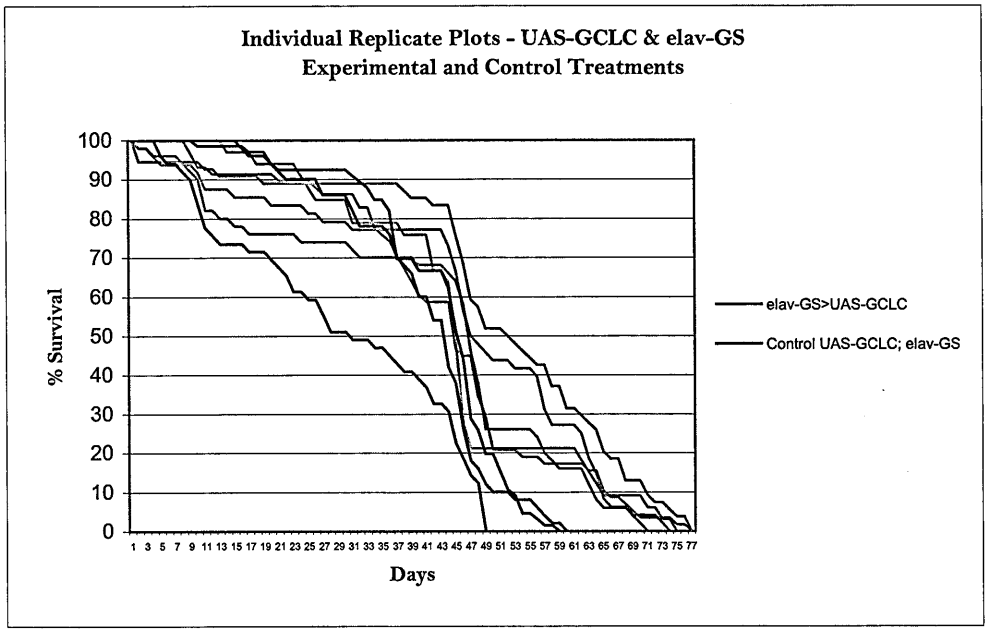


Table 5.6 Summary statistics for individual replicates from lifespan assay for male *UAS-GCLC/+; elav-GS/+* flies (experimental and control treatments)

Genotype	Treatment	Replicate	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value
UAS- GCLC/+; elav-GS/+	+RU486	1	58	42.97	2.10493	44	67	0.0436
		2	50	40.20	2.817	46	65	
		3	48	45.06	2.82865	46	66	
		4	33	44.18	2.59459	44	66	
		5	54	50.06	2.52637	52	72	
	-RU486	1	49	29.102	2.21702	30	50	<0.0001
		2	50	39.88	1.45143	43	51	
		3	66	41.95	1.2009	45	54	

When GCLC is over-expressed in a pan-neural pattern in male flies, it leads to a highly statistically significant increase in lifespan ($p=<0.0001$). Compared to control flies, *elav-GS* driven *UAS-GCLC* flies show an 18.77% increase in mean lifespan, a 6.98% increase in median lifespan and a 28.85% increase in maximum lifespan. Driven flies have a mean lifespan of 44.55 days, a median of 46 days and a maximum of 67 days compared to 37.51 days, 43 days and 52 days respectively for control flies (Figure 5.8 and Table 5.7). The fact that maximum lifespan is increased by a much higher percentage than median lifespan implies that the benefit of neural over-expression is in protection against senescence-related conditions.

Neither the experimental nor the control line demonstrates a true plateau phase although Phase I of the curve is demonstrably shallower than subsequent phases. This indicates that whilst there is a factor that impairs early survival, it is common to both treatments and therefore likely to be independent of manipulation of GCLC levels. The curve profiles are noticeably different between the experimental and control groups. The onset of ageing is later in the *elav-GS* driven *UAS-GCLC* flies (45 days in comparison to 30 days). In addition, in the driven flies Phase II (age-dependent mortality) is much shorter than in the control flies as the Phase III late-life plateau begins at approximately day 49 resulting in a markedly increased maximum lifespan (67 days in driven flies compared to 52 days in control flies). This implies that there is an advantage to lifetime over-expression of GCLC in neural tissue that becomes apparent in aged flies. The implications of this will be discussed in further detail in Section 5.4.3.

Figure 5.8 Lifespan assay for male *UAS-GCLC/+; elav-GS/+* flies (experimental and control treatments). Each dataset is pooled data from 3-5 replicate tubs run simultaneously. The curves show a statistically significant difference (log rank test, p=<0.0001).

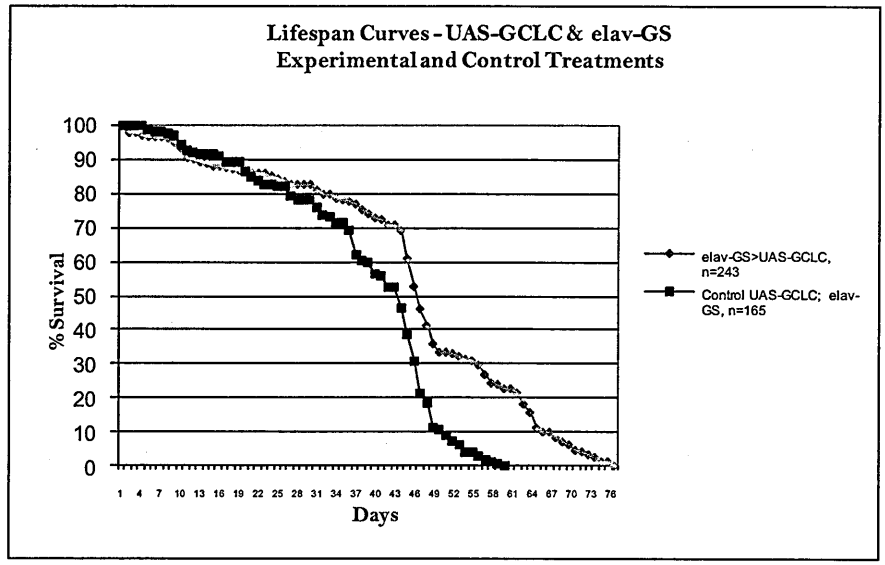


Table 5.7 Summary statistics for lifespan assay for male *UAS-GCLC/+; elav-GS/+* flies (experimental and control treatments). The percentage change figures for mean, median and maximum lifespan refer to the percentage change of driven flies relative to non-driven flies.

Genotype	Treatment	n (Flies)	Mean (days)	SEM	Median (days)	Max (90%) (days)	% Change Mean	% Change Median	% Change Max	Onset of Ageing (days)	Log Rank p value	DF
UAS- GCLM/+; elav-GS/+	+RU486	243	44.55	1.16827	46	67	↑ 18.77	↑ 6.98	↑ 28.85	45	<0.0001	1
	-RU486	165	37.51	1.01644	43	52				30		

Females

Initially, individual replicates were analysed to provide a statistical analysis of the spread of replicate curves (Figure 5.9 and Table 5.8). There was a statistically significant difference between the individual replicates. However, as experimental conditions were uniform and all replicates were fed on media from the same batch, replicates were pooled for the purpose of this analysis.

Figure 5.9 Individual replicates for lifespan assay for female *UAS-GCLC/+; elav-GS/+* flies (experimental and control treatments). Each replicate consists of an individual tub.

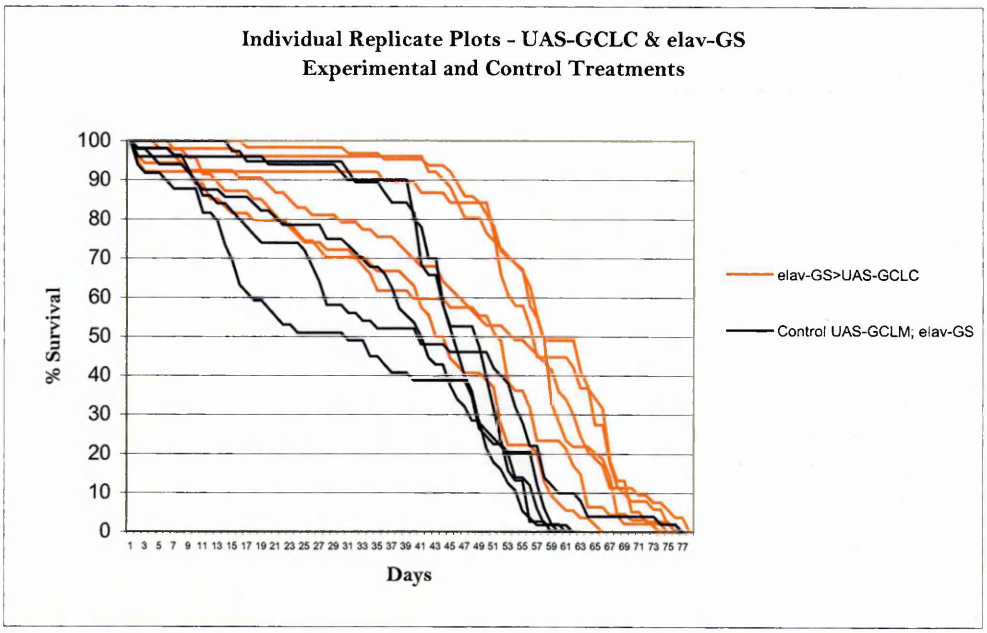


Table 5.8 Summary statistics for individual replicates from lifespan assay for female *UAS-GCLC/+; elav-GS/+* flies (experimental and control treatments).

Genotype	Treatment	Replicate	n (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value
UAS- GCLC/+; elav-GS/+	+RU486	1	54	39.07	2.44519	44	60	<0.0001
		2	47	42.60	2.82592	51	65	
		3	53	47.62	2.81328	53	72	
		4	64	55.75	1.21192	57	69	
		5	38	43.76	2.91473	56	71	
		6	51	56.63	1.89177	57	70	
	-RU486	1	56	36.79	2.05788	40	56	0.0363
		2	50	37.62	2.92699	40	61	
		3	49	30.59	2.86931	30	59	
		4	38	44.53	1.60358	48	57	
		5	50	43.72	1.63932	45	58	

When *UAS-GCLC* is over-expressed in female flies in neural tissue, a significant increase in mean lifespan is seen. This manifests as a 28.55% increase in mean lifespan, a 27.91% increase in median lifespan and a 18.97% increase in maximum lifespan (the driven flies have a mean lifespan of 49.30 days, a median of 55 days and a maximum of 69 days in comparison to 38.35 days, 43 days and 58 days in control flies) (Figure 5.10 and Table 5.9). As seen in male flies, there is no true plateau phase during Phase I of the curve, although there is a pronounced period of shallow slope in both experimental and control flies. However, compared to male flies, the experimental and control flies diverge much earlier in the assay at day 10. This is prior to the onset of age-dependent mortality and indicates that in female flies when GCLC is not over-expressed, there is higher age-independent mortality than their siblings where GCLC is over-expressed in neural tissue. The *elav-GS* driven *UAS-GCLC* flies show a later onset of ageing (50 days in comparison to 39 days in control flies). However, the rate of ageing is the same for both treatments. Again there is no pronounced shallowing of the curve later in the assay for female flies.

Figure 5.10 Lifespan assay for female *UAS-GCLC/+; elav-GS/+* flies (experimental and control treatments). Each dataset is pooled data from 5-6 replicate tubs run simultaneously. The curves show a statistically significant difference (log rank test, p=<0.0001).

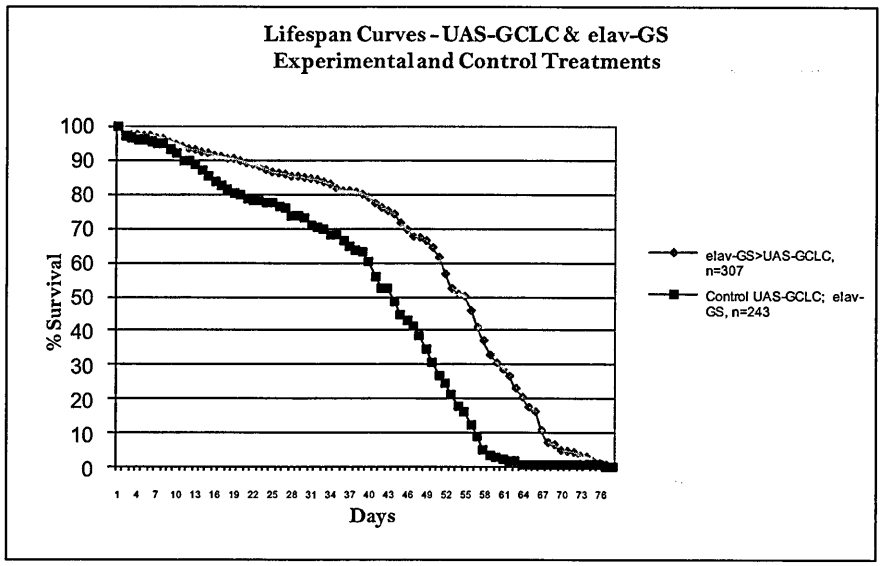


Table 5.9 Summary statistics for lifespan assay for female *UAS-GCLC/+; elav-GS/+* flies (experimental and control treatments). The percentage change figures for mean, median and maximum lifespan refer to the percentage change of driven flies relative to non-driven flies.

Genotype	Treatment	n (Flies)	Mean (days)	SEM	Median (days)	Max (90%) (days)	% Change Mean	% Change Median	% Change Max	Onset of Ageing (days)	Log Rank p value	DF
UAS- GCLM/+; elav-GS/+	+RU486	307	49.30	1.01745	55	69	↑28.55	↑27.91	↑ 18.97	50	<0.0001	1
	-RU486	243	38.35	1.08792	43	58				39		

5.3.3 Co-Overexpression of GCLM and GCLC

Males

Initially, individual replicates were assessed for spread and were shown to be statistically significantly different from each other. These replicates formed three distinct experimental blocks, run at separate times. Individual replicates within each block were pooled for the purpose of this analysis as they were run simultaneously and fed on media from the same batch. However, each experimental block was analysed separately as, although every effort was made to ensure consistency between experimental runs, it is impossible to rule out minor differences in conditions which could have affected lifespan. Full details of all replicate lines are listed in Table 5.10. Experimental Block 1 is discussed in detail below as a representative example of these repeat assays. Both experimental and control lines showed a steep initial decline post-anaesthesia. This was the only experimental block to show this and the fact that both the experimental and control flies showed this decline indicates that it is possible that it is purely an adverse reaction to collection and anaesthesia. In order to remove this confounding factor from the analysis, data prior to day 5 of the analysis was censored. Graphs are shown post-censor and the number of censored flies is detailed in Table 5.10 and Table 5.11.

Table 5.10 Summary statistics for individual replicates from lifespan assays for male *UAS-GCLC,GCLM/+; elav-GS/+* flies (experimental and control treatments). Each replicate consists of an individual tub. Each experimental block consists of one set of experimental replicates and one set of control replicates run simultaneously, under identical conditions, fed on media from the same batch.

Experimental Block	Treatment	Replicate	n (Flies)	Cen (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value
1	+RU486	1	48	2	41.96	1.97972	42	62	<0.0001
		2	48	5	42.71	1.70288	43	57	
		3	46	3	36.91	2.36517	43	55	
		4	45	3	34.76	2.18145	39	50	
		5	44	3	39.98	2.36596	45	59	
		6	48	1	44.19	1.62937	43	61	
		7	49	0	42.04	1.98227	45	61	
		8	43	6	36.16	2.11594	39	52	
		9	47	2	28.89	2.14878	31	50	
		10	47	5	37.96	1.78076	40	50	
	-RU486	1	49	0	27.78	1.52843	28	49	<0.0001
		2	27	2	34.41	1.69905	37	48	
		3	47	2	37.57	1.75559	42	50	
		4	48	2	27	1.96873	23	48	
		5	39	1	26.67	2.07463	24	47	
		6	47	2	33.98	1.80232	39	48	
		7	46	3	29.65	2.304	34	51	
		8	49	1	27.20	1.71056	33	44	
		9	37	3	27.30	2.34356	31	45	
		10	45	4	31.73	2.34616	37	52	
		11	43	2	34.88	2.02482	38	51	
2	+RU486	1	31	0	28.87	2.50193	32	46	0.0023
		2	75	0	30.83	2.04326	29	55	

		3	36	0	37.94	4.10225	40	71	
	-RU486	1	34	0	26.24	2.10553	27	45	0.0011
		2	75	0	18.32	1.14154	17	34	
		3	36	0	21.92	1.73954	23	37	
3	+RU486	1	73	0	34.92	1.70065	35	54	0.0028
		2	70	0	31.4	1.38842	34	48	
	-RU486	1	74	0	25.27	1.28879	26	40	0.7547
		2	74	0	25.43	1.14257	23	42	

When both sub-units are over-expressed simultaneously in a pan-neural pattern, a significant lifespan extension is seen (Figure 5.11 and Table 5.11). This improvement in survival is replicated in each experimental block. Driven flies in Experimental Block 1 have a 25.99% higher mean lifespan, a 23.53% higher median lifespan and a 16.33% higher maximum lifespan when compared to control lines (driven flies have a mean lifespan of 38.63 days, a median of 42 days and a maximum of 57 days in comparison to 30.66 days, 34 days and 49 days respectively for control flies).

Figure 5.11 shows a representative curve for these data taken from Experimental Block I. In both experimental and control lines, there is a small initial plateau phase followed by a steepening of the curves, although there is no clear Phase I plateau as was seen in the previous assays. Divergence between experimental and control lines occurs at day 10 - at this point, the curve for driven flies has a second period of shallowing which is much more pronounced than that of the control flies. Whilst Phase II of both curves (the portion representing age-dependent mortality) is similar in slope between treatments, implying that there is little advantage to the flies during this period of over-expression of GCLC and GCLM together, there is once again a reduction in mortality during Phase III of the curve for driven flies that is not seen in control groups, implying that there is a late survival advantage for driven flies of co-overexpression of GCL subunits. It is not possible to reliably estimate the onset of ageing for these data as a result of the two-step steepening that occurs.

Figure 5.11 Representative lifespan assay for male *UAS-GCLC,GCLM/+; elav-GS/+* flies (experimental and control treatments). Each dataset is pooled data from 10-11 replicate tubs from Experimental Block 1. The curves show a highly statistically significant difference (log rank test, p=<0.0001).

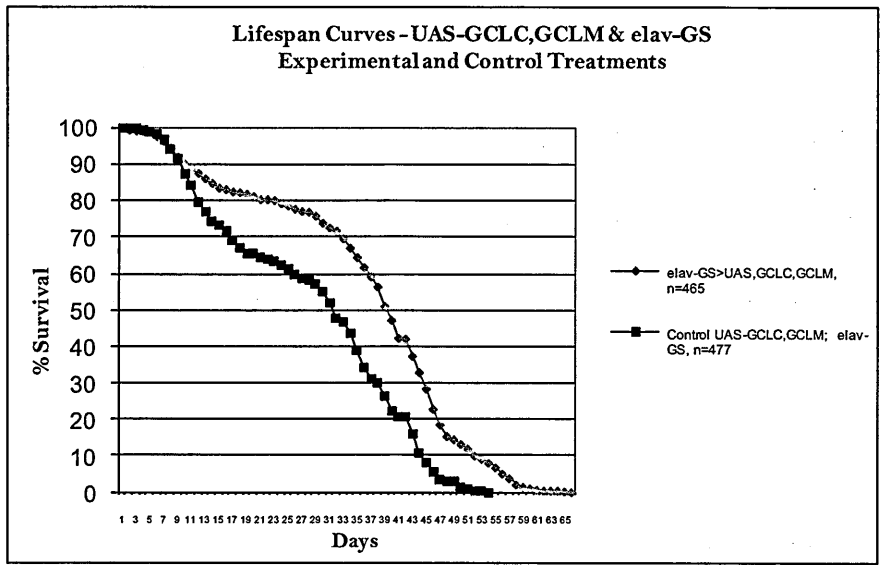


Table 5.11 Summary statistics for all lifespan assays for male *UAS-GCLC,GCLM/+; elav-GS/+* flies (experimental and control treatments). Each experimental block consists of one set of experimental replicates and one set of control replicates run simultaneously, under identical conditions, fed on media from the same batch. The percentage change figures for mean, median and maximum lifespan refer to the percentage change of driven flies relative to non-driven flies. The estimated onset of ageing has not been included in this table. The reasons for this are discussed in Section 5.3.3.

Experimental Block	Treatment	n (Flies)	Cen (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	% Change Mean	% Change Median	% Change Max	Log Rank p value	DF
1	+RU486	465	30	38.63	0.66754	42	57	↑ 25.99	↑ 23.53	↑ 16.33	<0.0001	1
	-RU486	477	22	30.66	0.61725	34	49					
2	+RU486	142	0	32.20	1.60954	32	61	↑ 52.82	↑ 52.38	↑ 60.53	<0.0001	1
	-RU486	145	0	21.07	0.916	21	38					
3	+RU486	143	0	33.20	1.10857	35	52	↑ 30.97	↑ 52.17	↑ 26.83	<0.0001	1
	-RU486	148	0	25.35	0.85826	23	41					

Females

Initially, individual replicates were assessed for spread and were shown to be statistically significantly different from each other. As with male flies, these replicates formed three distinct experimental blocks, run at separate times. Individual replicates within each block were pooled for the purpose of this analysis as they were run simultaneously and fed on media from the same batch. However, each experimental block was analysed separately for the reasons cited above for male flies. Full details of all replicate lines are listed in Table 5.12. Experimental Block 1 is discussed in detail below as a representative example of these repeat assays. As with male flies described above, both experimental and control lines showed a steep initial decline post-anaesthesia and therefore the data shown below are censored for mortality prior to day 5 in order to reduce complicating variables. Graphs are shown post-censor and the number of censored flies is detailed in Table 5.12 and Table 5.13.

Table 5.12 Summary statistics for individual replicates from lifespan assay for female *UAS-GCLC,GCLM/+; elav-GS/+* flies (experimental and control treatments). Each replicate consists of an individual tub. Each experimental block consists of one set of experimental replicates and one set of control replicates run simultaneously, under identical conditions, fed on media from the same batch.

Experimental Block	Treatment	Replicate	n (Flies)	Cen (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	Log Rank p value
1	+RU486	1	45	3	48.49	1.95133	52	66	<0.0001
		2	47	2	50.77	1.68363	52	66	
		3	34	2	47.35	2.34497	51	63	
		4	46	2	45.28	1.75303	47	61	
		5	44	4	42.23	2.12427	44	61	
		6	44	3	43.32	1.7511	46	57	
		7	45	3	46.09	1.50683	47	60	
		8	41	6	46.27	1.41207	46	60	
		9	35	11	38.77	2.18862	39	59	
	-RU486	1	48	3	42.06	0.98049	48	51	<0.0001
		2	45	5	38.76	1.36299	45	51	
		3	44	4	38.02	1.52412	44	53	
		4	33	2	39.94	1.41618	33	48	
		5	42	6	40.64	1.45383	42	53	
		6	45	5	47.13	1.70845	45	61	
		7	47	4	38.43	1.74024	47	54	
		8	46	4	47.39	1.71311	46	62	
		9	41	9	45.85	1.58943	41	59	
2	+RU486	1	33	0	36.06	2.51696	38	52	0.0011
		2	74	0	27.97	1.5997	29	49	
		3	39	0	35.23	3.06684	35	62	
	-RU486	1	34	0	27.76	2.45024	25	47	0.5970

		2	73	0	27.37	1.51106	28	45	
		3	40	0	28.78	2.56867	24	54	
3	+RU486	1	60	0	36.13	1.53186	36	53	<0.0001
		2	63	0	26.335	1.37998	26	42	
	-RU486	1	75	0	32.84	1.46491	31	47	0.0925
		2	75	0	36.09	1.55059	40	53	

When both sub-units are over-expressed simultaneously in a pan-neural pattern, a statistically significant lifespan extension was seen in Experimental Blocks 1 and 2 (Figure 5.12 and Table 5.13). However, Experimental Block 3 showed a modest but statistically significant *decrease* in mean and median lifespan (a 9.8% decrease in mean lifespan, an 8.33% decrease in median lifespan) although maximum lifespan in the group of flies co-overexpressing both subunits is still increased by 1.92%. This inability to replicate extension in all cases indicates that either the extension seen in driven female flies is inherently variable or that it is sensitive enough to small deviations in experimental conditions between runs to be reversed under certain conditions.

Figure 5.12 shows a representative curve for the experimental blocks that show extension (in this case, data from Experimental Block 1). It is evident from these data that the divergence occurs during Phase II of the curve. Both driven and non-driven flies have an onset of ageing of 32 days. It is in Phase II of the curve (where mortality is age-dependent) that the difference between experimental and control flies occurs, with experimental flies 'ageing' slower, or showing a shallower curve slope. This leads to an ultimate extension in maximum lifespan (61 days in comparison to 58 days for the control flies). Again, as has been seen previously with female flies, there is no late shallowing of the curve in Phase II and therefore no evidence of a later life advantage to these lines of over-expression of the subunits together.

Figure 5.12 Representative lifespan assay for female *UAS-GCLC,GCLM/+; elav-GS/+* flies (experimental and control treatments). Each dataset is pooled data from 9 replicate tubs from Experimental Block 1. The curves show a statistically significant difference (log rank test, p=<0.0001)

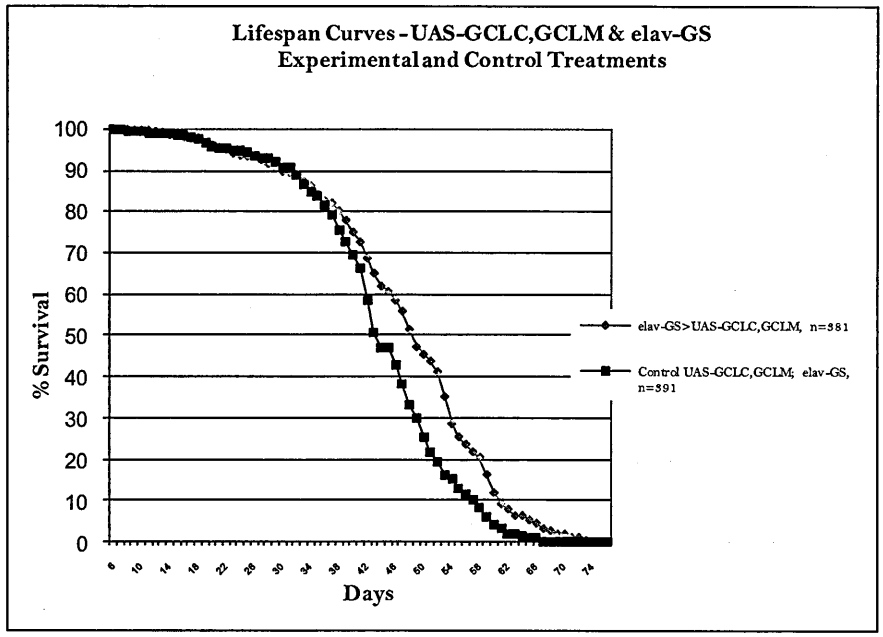


Table 5.13 Summary statistics for all lifespan assays for female *UAS-GCLC,GCLM/+; elav-GS/+* flies (experimental and control treatments). The percentage change figures for mean, median and maximum lifespan refer to the percentage change of driven flies relative to non-driven flies. The estimated onset of ageing has not been included in this table. The reasons for this are discussed in Section 5.3.3. Each experimental block consists of one set of experimental replicates and one set of control replicates run simultaneously under identical conditions, fed on media from the same batch.

Experimental Block	Treatment	n (Flies)	Cen (Flies)	Mean (Days)	SEM	Median (Days)	Max (90%) (Days)	% Change Mean	% Change Median	% Change Max	Log Rank p value	DF
1	+RU486	381	36	45.55	0.63366	47	61	↑ 8.27	↑11.90	↑5.17	<0.0001	1
	-RU486	391	42	42.07	0.5351	42	58					
2	+RU486	146	0	31.74	1.31476	32	52	↑ 14.01	↑ 18.52	↑6.12	0.0153	1
	-RU486	147	0	27.84	1.16405	27	49					
3	+RU486	123	0	31.12	1.11594	33	51	↓ 9.80	↓ 8.33	↑1.92	0.0116	1
	-RU486	153	0	34.50	1.07258	36	52					

5.3.4 Enhanced Neural Glutathione Titres and Lifespan Extension

Results presented in Section 5.3.3 indicate that there is a survival advantage when the GCLC and GCLM subunits are co-overexpressed in neural tissue throughout the adult lifespan of flies in a wild-type background, providing an increase in expression above endogenous levels. This supports the hypothesis that neural tissue is lifespan limiting and that damage to this tissue is responsible for age-dependent mortality in *Drosophila* under normal conditions. The question remains as to whether this advantage persists under conditions of increased oxidative stress. In order to investigate this, flies of the genotype *w*; *UAS-GCLC,GCLM*/+; *elav-GS*/+ were exposed to two chemical stressors in the presence of RU486 in the dosing solution (driven) and in the absence of RU486 in the dosing solution (non-driven). As discussed in Section 1.7.4, chemical stressors can be used to induce oxidative imbalance in *Drosophila.* In order to investigate the relationship between increased GCL levels in neural tissue and survival under conditions of increased oxidative insult, flies were exposed to paraquat and DEM. Paraquat creates a direct oxidative stress via the production of $O_2^{\bullet -}$, whilst DEM acts to deplete glutathione itself.

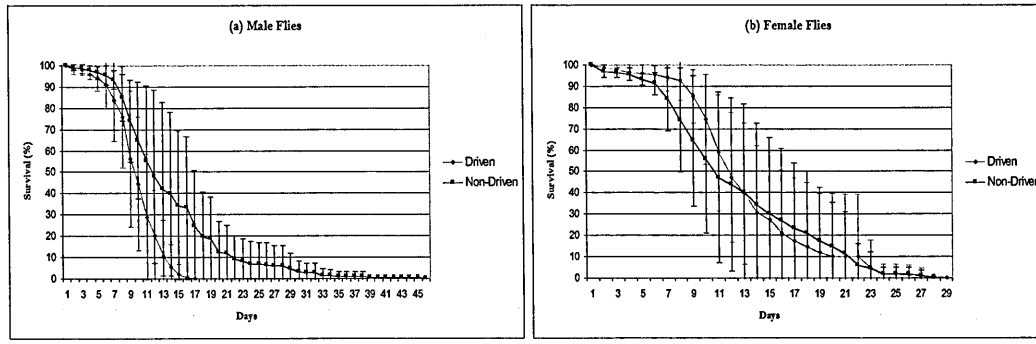
DEM Treatment

When male *w*; *UAS-GCLC,GCLM*/+; *elav-GS*/+ flies are fed on a 5mM DEM and sucrose solution, there is a statistically significant difference between survival times for the group of driven and the group of non-driven flies (Figure 5.13a). However, this manifests as a

survival impairment for flies where GCLC and GCLM are co-overexpressed in a pan-neural pattern, contrary to predictions based on the advantage that over-expression provides under normal conditions (Section 5.3.3). Driven flies show reduced mean, median and maximum survival (9.04 days, 9 days and 13 days respectively) when compared to their non-driven siblings (non-driven flies have a mean survival of 13.16 days, a median of 11 and a maximum of 21 days).

This effect is not replicated in female flies (Figure 5.13b) where there is no significant difference between driven and non-driven flies. Driven flies have a median lifespan of 12.40 days, a median of 11 days and a maximum of 20 days, in comparison to 11.79 days, 10 days and 21 days respectively for their non-driven siblings. The individual replicates for female flies were inherently far more variable than their male counterparts, possibly as a result of the complicating factor of egg production and laying and the metabolic cost that this exerts.

Figure 5.13 DEM exposure assay for (a) male and (b) female flies. *UAS-GCLC,GCLM/+; elav-GS/+* flies were exposed to daily doses of 5mM DEM in a 10% sucrose solution with (driven) and without (non-driven) RU486. Each graph represents pooled data from between 4 and 8 replicate tubs, run simultaneously under identical conditions. The survival curves for male flies are highly statistically significantly different (p=<0.0001, log rank test). The survival curves for female flies are not statistically significantly different (p=0.2744, log rank test).

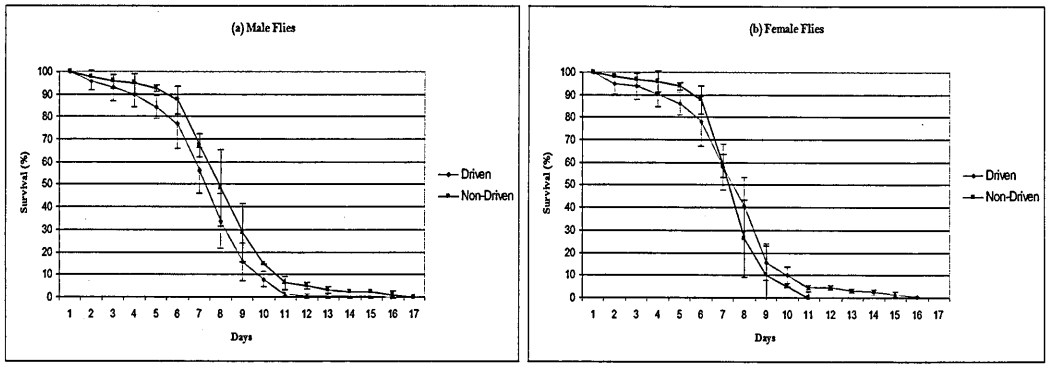


Paraquat Treatment

When male *w*; *UAS-GCLC,GCLM/+*; *elav-GS/+* flies are fed on a 5mM paraquat and sucrose solution, there is again a highly statistically significant impairment of stress resistance, as indicated by survival times of driven and non-driven sibling flies (Figure 5.14a). Driven flies have a mean lifespan of 6.57 days, a median of 7 days and a maximum of 9 days in comparison to 7.47 days, 7 days and 10 days respectively for non-driven flies. Despite statistical significance, these increases are minor with no difference in median lifespan and small differences in mean and maximum lifespan.

Again, this statistically significant difference is not replicated with female flies with no significant difference between driven and non-driven flies (Figure 5.14b). As with the DEM exposure assay, female flies showed a higher variability between replicates, possibly a result of the additional complicating factor of egg production and laying.

Figure 5.14 Paraquat exposure assay for (a) male and (b) female flies. *UAS-GCLC,GCLM/+; elav-GS/+* flies were exposed to daily doses of 5mM paraquat in a 10% sucrose solution with (driven) and without (non-driven) RU486. Each graph represents pooled data from between 2 and 4 replicate tubs, run simultaneously under identical conditions. The survival curves for male flies are highly statistically significantly different (p=<0.0025, log rank test). The survival curves for female flies are not statistically significantly different (p=0.1094, log rank test).



Stress Resistance in Neural Tissue

These results are contrary to predictions made based on the lifespan results presented in Section 5.3.3 and are also contrary to predictions made based on the modes of action of the chemical stressors used.

As DEM directly depletes glutathione levels, thereby creating an environment where oxidative stress is likely to be higher, it would be expected that an increase in levels of both the subunits of the rate limiting enzyme in the glutathione synthesis pathway would facilitate further synthesis to replace the depleted glutathione hence maintaining a higher level of production compensating for the depletion caused by DEM exposure. Under these circumstances, flies where over-expression of both GCL subunits is driven in neural tissue would have an advantage over non-driven sibling flies which could lead to improved survival. As the opposite is seen in male flies (Figure 5.14a), it is possible that glutathione synthesis in neural tissue is limited by more than simply GCL subunit levels. It is possible that substrate availability, particularly glutamate levels, limits synthesis in neural tissue which would explain an absence of extension when subunits are over-expressed.

However, it is more difficult to explain why statistically significant extension is seen in non-driven flies. This implies that over-expression of GCL subunits in neural tissue is actually detrimental to survival under conditions of elevated oxidative stress. The fact that DEM could be exerting toxic effects unrelated to the perturbation of oxidative balance is also

possible, although that would not account for the increased survival seen in male flies without subunit over-expression. Whilst RU486 has no discernible effect on survival in male flies (see Figure 5.3), it is possible that it is exerting an effect that makes flies more susceptible to oxidative stressors, however, if this is the case, it appears to be sex-specific as the effect is not replicated in female flies.

It must be borne in mind that this stress is globally delivered whilst over-expression is localised to neural tissue. Therefore, these results suggest that flies' oxidative defence enzymes already function at optimal levels and that any deviation from those levels, under conditions of oxidative stress, leads to impairment of the flies' ability to handle oxidative insult. Nonetheless, it still supports the hypothesis that neural tissue is a lifespan limiting tissue in flies as manipulation of GCL levels in this tissue alone is sufficient to cause impairment under conditions of globally elevated oxidative stress.

Male flies also show statistically significantly impaired survival when GCL subunits are co-overexpressed in neural tissue when flies are exposed to paraquat, a chemical that generates oxidative stress via the generation of $O_2^{\bullet -}$. This indicates that perturbation of GCL subunit levels exerts an effect beyond the direct effect seen in the presence of a glutathione depletor such as DEM. It is possible that this is via interactions with other components of the oxidative defence system such as SOD (Orr *et al* 1998).

5.4. Discussion

5.4.1 GCLM

Contrary to predictions based on the lack of independent catalytic activity of the modifier subunit, over-expression of GCLM actually leads to a highly statistically significant increase in lifespan when expressed in neural tissue. This raises the question of how GCLM over-expression could affect longevity in such a pronounced fashion without exerting individual catalytic effect.

GCLC and GCLM interact covalently via the formation of disulphide bridges (Fraser, Kansagra et al, 2003). Under these circumstances, GCLM improves the catalytic efficiency of GCLC, specifically lowering the K_m for the substrate glutamate, i.e. GCLC gains a higher affinity for the substrate (Fraser, Saunders et al. 2002; Fraser, Kansagra et al. 2003). If glutamate availability is limiting in neural tissue, this increased affinity resulting from an excess of available GCLM in over-expressing flies could account for the increases in lifespan seen in driven flies versus control lines. Onset of ageing is later in male and female elav-GS driven lines and this implies that a key factor in the timing of the onset of the age-dependent mortality phase of these flies' lifespans could be neural degeneration due to oxidative damage – up-regulation of the oxidative defence system results in a rightward shift of this point on the lifespan curves and hence a delay in the timepoint when this damage becomes lifespan limiting.

There is a distinct difference between male and female flies' response to this overexpression – they age differently which implies that different effects come into play in each sex. This will be discussed in more detail in Section 5.4.4 below.

These data once again contradict those of Orr and colleagues (Orr, Radyuk et al. 2005), who saw statistically significant extension when GCLM was over-expressed globally but not when GCLM was over-expressed in the CNS (Orr, Radyuk et al. 2005). There is a fundamental difference between the elav driver used in this case and that used by Orr *et al*: the elav-GS driver used in the experiments described in this Chapter enables transgene expression to be restricted to the adult phase of the fly's life. Orr and colleagues used an elav-GAL4 driver that is constitutively expressed from embryogenesis onwards. There are two reasons why this could lead to a less accurate picture of the effects of over-expression on adult lifespan. The first is that developmental effects of over-expression could cause changes that affect long-term adult survival. This is certainly possible as it has been shown in this thesis that high-levels of developmental global over-expression in pre-adult developmental stages has a severe developmental effect (Chapter 3). The fact that the elav-GAL4 driven flies described by Orr *et al* (2005) do not appear to show the same extreme lethality phenotype does not necessarily mean that there are not more subtle effects that manifest as lifespan impairment or negate any later-life positive effects of over-expression rather than overt lethality. In addition, as the GAL4 expression system requires the combination of chromosomes from the driver and responder elements, there is the possibility that genetic background could influence the eventual longevity results. Orr *et al* isogenised their fly lines by backcrossing for 6 generations but as the data presented in

Chapters 3 and 4 show, it is still possible that genetic differences may persist despite backcrossing and these can have a profound effect on longevity. The use here of the Geneswitch system bypasses this variable as expression is induced via delivery of a synthetic progesterone hormone in the food media once the flies have reached 2 days of age.

5.4.2 GCLC

The data presented in Section 5.3 above supports the hypothesis that over-expression of GCLC, which has independent catalytic activity, exerts a protective effect that is beneficial to longevity. Over-expression of GCLC results in a delayed onset of ageing, a shorter period when mortality rate is highest and, in male flies, a very pronounced reduction in mortality in later life. Female flies also show enhanced early survival, delayed onset of ageing but no late reduction in mortality (the implications of these differences is discussed in Section 5.4.4). This supports the hypothesis that oxidative stress in neural tissue is a defining factor in the timing of the onset of ageing, although less of a factor in the determination of the rate of ageing. This implies that neural tissue condition is limiting to a point but that once past that point, other factors become lifespan limiting. It is possible that as a fly ages, the situation changes and in later life, at least for male flies, neural tissue condition again becomes a limiting factor in survival.

These data concur with those of Orr *et al* (2005), who describe significant extension of mean lifespan of 24.0% and 43.4% in two separate insertion lines of driven flies relative to

driver control flies. However, Orr and colleagues report that one insertion line actually shows a statistically insignificant decrease in mean lifespan relative to controls (Orr *et al.* 2005). This inconsistency exemplifies the variation that can be inherent between different P-element insertion lines.

5.4.3 The Holoenzyme Complex

When GCLC and GCLM are over-expressed simultaneously in using the elav-GS driver, the lifespan curve show a different relationship entirely between experimental and control lines to the individual sub-units alone. In this case, the rate of ageing is reduced but onset is not affected in male and female flies. There is still an improvement in late-life survival in male flies but this is much less marked than when GCLM and GCLC are over-expressed alone. This raises the question as to why co-over-expression would lead to effects in different sections of the survival curves.

It is likely that when GCLM is over-expressed in a wild-type background, most of the endogenous GCLC will be bound to GCLM and will, therefore, demonstrate a higher efficiency for glutamate. There may be unbound GCLM present. When GCLC is over-expressed in a wild-type background, it is possible that there will be a mixture of bound and unbound GCLC thus a variety of different affinities for glutamate but a greater catalytic arsenal. When both are co-over-expressed in a recombinant fly, potentially all the additional GCLC could be bound to GCLM, hence providing a greater catalytic arsenal

with increased substrate affinity. It is not clear how this could lead to a difference in where the survival advantage occurs. The presence of two P-element insertions in the recombinant line does increase the potential for deleterious position effects that could affect how these flies age, independent of over-expression, so it is possible that the distinct ageing profiles are a result of factors independent of GCL subunit ratios. Nonetheless, significant lifespan extension is seen in both cases which supports the hypothesis that oxidative protection in neural tissue is a key modifier of longevity. Orr and colleagues did not assay a fly line expressing both GCL subunits and, therefore, no direct comparison can be made.

These results demonstrate that over-expression of GCL in neural tissue during a fly's lifespan can have an effect on the longevity of a particular strain. It is also clear that there are key stages in the lifespan of these flies where this exerts an effect. It would be interesting, given the temporal flexibility of the Geneswitch system, to examine whether a similar mortality phenotype occurs if expression is induced later in the life of the flies and if the advantage gained by early over-expression persists even if expression is not induced past a certain point in the assay. It would also be interesting to examine whether bypassing the developmental phase by using a tubulin or actin Geneswitch driver leads to extension of longevity.

The data presented here suggest that there is no link between increased longevity due to tissue-specific over-expression of GCL under normal conditions and increased ability to deal with specific oxidative insult when global oxidative balance is perturbed. A deviation

from endogenous levels of GCL impairs resistance to DEM treatment (a glutathione depletor) and, to a lesser extent, resistance to paraquat exposure. This implies that stress response mechanisms respond differently under normoxic conditions than under conditions of elevated stress. The implication from the stress resistance assays and lifespan data reported in this chapter is that stress response mechanisms respond differently under conditions of elevated stress. It would appear that the endogenous levels of GCL are optimal to cope with increased oxidative insult but are sub-optimal under conditions of normal oxidant exposure associated with the ageing process. This neither supports nor contradicts the hypothesis that increased levels of antioxidants provide an advantage that increases lifespan. Instead, these results suggest that the relationship between oxidative stress protection and ageing is more complex than a simple cause and effect relationship, with external factors such as ambient levels of oxidative stress exerting an effect.

Ambient conditions are not the only factor that influences survival in flies. Whilst the stress assays presented here, using young flies, show no advantage to over-expression of GCL in handling increased oxidative insult, Orr and colleagues (2005) report a significant increase in stress resistance when GCLC is over-expressed in aged flies. This implies that the relative importance of oxidative damage protection shifts as a fly ages. Taking this into account, it would be interesting to examine whether late-life induction of GCL over-expression also provides an advantage or whether this is a result of an accumulation of protective effects throughout the flies' lifespan that manifests as an increase in late-life stress resistance.

Both the positive stress resistance result that Orr and colleagues (2005) reported and the negative results that are discussed in this chapter support the hypothesis that oxidant damage to neural tissue plays a key role in organismal longevity as perturbations of GCL levels in neural tissue alone are sufficient to induce significant changes in the fly's ability to handle increased levels of oxidative stress.

5.4.4 Sex-Specific Differences in Response to Over-Expression of GCL

The additional metabolic load that females are exposed to as a result of egg production and laying means that male and female lifespans are not directly comparable. Female *Drosophila melanogaster* are also subject to a negative cost attributed to mating with male flies, resulting from exposure to seminal fluid products from male accessory glands (Chapman, Partridge et al, 1995). Nevertheless, it is interesting to note that, whilst both sexes show statistically significant extension, there is a difference in the profile of the curves implying that the relationship between lifespan, glutathione levels and sex is complex. In female flies, it appears that increased protection during early adulthood, when egg production and the associated metabolic cost are highest, gives rise to increased lifespan across the population studied. In male flies, there appears to be an additional advantage that manifests as improved late-life survival.

All of the results above exemplify the need to examine the connection between oxidative stress response and ageing in the context of internal factors (e.g. tissue-specific expression

profiles), external factors (e.g. external redox environment) and factors such as the sex and age of the population being studied. It is only through considering results in the context of all contributing factors, that the relationship between antioxidant enzyme expression, ageing and stress response can be clearly elucidated.

6. DISCUSSION

6.1. The Relationship Between Glutathione and Lifespan Extension

The free radical theory of ageing proposes that organismal ageing and physiological decline are a result of age-related accumulation of oxidative damage caused by oxidants generated as a result of both endogenous and exogenous factors (Harman, 1956). In recent years, a sizeable body of work has been published supporting the oxidative damage theory of ageing (Arking 1987; Arking, Buck et al. 1988; Parkes, Elia et al. 1998; Arking, Burde et al. 2000; Arking, Burde et al. 2000; Mockett, Orr et al. 2001; Orr, Mockett et al. 2003; Landis, Abdueva et al. 2004; Orr, Radyuk et al. 2005; Luchak, Prabhudesai et al. 2007). However, in recent years external factors such as genetic background have been implicated as the driving force behind increases in lifespan seen as a result of manipulation of levels of SOD (Orr and Sohal 2003) and a variety of other genes and treatments have also been demonstrated to affect longevity (Goyns and Lavery 2000; Clancy, Gems et al. 2001; Rogina, Helfand et al. 2002; Ruan, Tang et al. 2002; Tatar, Bartke et al. 2003; Giannakou, Goss et al. 2004). It is now commonly accepted that ageing is a polygenic phenomenon and that it is affected by a diverse range of conditions and treatments. This thesis aimed to further investigate the link between organismal oxidative balance and ageing.

As a major cellular antioxidant, glutathione was identified as a potential target for interventions which could positively impact organismal longevity. Organismal ageing is accompanied by a pro-oxidising shift in the GSH:GSSG ratio, a marker of elevated

oxidative stress which, in *Drosophila*, results from an increase in GSSG rather than the depletion of the GSH pool that is seen in mice (Sohal, Mockett et al. 2002; Rebrin, Bayne et al. 2004). In addition, H_2O_2 production is increased (Sohal, Mockett et al. 2002). This shift is even more pronounced in the mitochondria, making them more susceptible to age-related glutathione depletion (Rebrin, Kamzalov et al. 2003). Chemical or genetic depletion of glutathione has serious cellular consequences resulting in mitochondrial damage and permeability, eventually leading to cell death (Meister 1995; Armstrong and Jones 2002; Valverde, Rojas et al. 2006). For these reasons, the primary aim of this thesis was to investigate the physiological consequences of manipulations that affect glutathione titres in *Drosophila melanogaster*, focusing on the effects on lifespan. A further objective was to investigate whether any increase in lifespan was accompanied by an increase in resistance to oxidative stress, thereby providing evidence of a link between increased longevity and increased ability to deal with oxidative insult.

Three factors combine to regulate glutathione homeostasis *in vivo*: the rate of GSH synthesis, the rate of GSH utilisation and the rate of GSH export (Griffith 1999). This thesis primarily focused on one of these parameters, the rate of synthesis. The rate limiting enzyme in GSH synthesis, GCL, was identified as a key manipulation point. Alongside a reduction in GSH affinity for substrates, ageing is accompanied by an over-all reduction in GCL catalytic activity and gene expression (Squier 2001; Toroser, Yarian et al. 2006; Toroser, Orr et al. 2007). The primary objective of this study was to investigate the physiological consequences of over-expression of this enzyme in relation to both longevity and stress resistance. It was hypothesised that an increase in GCL levels would compensate

for the age-related reduction in catalytic activity and gene expression, manifesting in an increase in longevity and stress resistance.

GCL is a heterodimer comprising a 73kDa catalytic subunit (GCLC) and a 31kDa modifier subunit (GCLM) (Huang, Chang et al. 1993; Misra and Griffith 1998; Tu and Anders 1998; Griffith 1999; Yang, Dieter et al. 2002). Whilst the catalytic subunit has independent catalytic activity, the modifier sub-unit does not, instead it functions to increase GCLC catalytic activity on binding and forming a holoenzyme complex by increasing GCLC's affinity for its substrates and reducing its susceptibility to feedback inhibition from GSH (Griffith 1999; Fraser, Saunders et al. 2002; Yang, Dieter et al. 2002). GCLC is vital to organismal survival with mutations in *Gclc* associated with human disease and null mutations in mice leading to embryonic lethality and post-embryonic ablation leading to death (Dalton, Dieter et al. 2000; Ristoff, Augustson et al. 2000; Shi, Osei-Frimpong et al. 2000; Chen, Yang et al. 2007; Mañú-Pereira, Gelbart et al. 2007). The absence of GCLM, in contrast, has no overt effect on survival and viability (Yang, Dieter et al. 2002; Fraser, Kansagra et al. 2003; McConnachie, Mohar et al. 2007). Nonetheless, GCLM has been proposed as the limiting factor for holoenzyme formation (Krzywanski, Dickinson et al. 2004; Chen, Shertzer et al. 2005; Lee, Kang et al. 2006; Franklin, Backos et al. 2009). Absence of GCLM in fibroblasts leads to premature senescence and it has been identified as the key GCL subunit in observed increases in GCL activity and elevated GSH titres in astrocytes and neurons (Chen, Johansson et al. 2009; Lavoie, Chen et al. 2009).

This study aimed to determine the effect of manipulation of levels of both GCL subunits, in isolation and co-overexpressed, in order to fully elucidate their roles in lifespan extension. The first objective of this study was to investigate the effect of a global upregulation of GCL subunits on lifespan. The subunits were over-expressed ubiquitously using the GAL-4-UAS system. Work by Orr and colleagues has previously demonstrated that ubiquitous over-expression of GCLM leads to a significant increase in lifespan (Orr, Radyuk et al. 2005). This thesis did not corroborate these findings, with no significant increase in longevity being seen when the modifier subunit was over-expressed without commensurate over-expression of the catalytic subunit. These results imply that the modest increases in lifespan associated with GCLM over-expression reported previously are possibly affected by culture conditions and handling and that it is not a robust extension. Furthermore, any lifespan differences were highly sensitive to the genetic background of the fly strains used. Isogenisation via backcrossing was shown to have limitations and did not entirely remove background variation from the strains used here. This exemplified the necessity of using an expression system in lifespan studies that bypasses this issue by over-expressing the gene of interest in genetically identical strains to control flies. Data presented here from the ubiquitous over-expression of the catalytic subunit alone do not support the hypothesis that a ubiquitous increase in GCL levels and hence organismal glutathione titre would lead to increased longevity as a result of a reduction in oxidative damage. In contrast, data presented here show that an increase in GCLC levels ubiquitously is detrimental to organismal survival, with a reduction seen in mean, median and maximum lifespan. This corroborates results published by another study where ubiquitous GCLC over-expression reduced lifespan in two out of three insertion lines (Orr,

Radyuk et al. 2005). Analysis of the mortality data presented in this thesis shows that this significant reduction in longevity results from an increase in post-developmental non age-dependent mortality indicating that there are consequences of the developmental over-expression of GCLC which affect adult longevity. Co-overexpression of GCLC and GCLM ubiquitously gave rise to an even more extreme lethality phenotype and this will be discussed further in Section 6.3.

The oxidative damage hypothesis predicts that increases in longevity associated with the protection from age-related oxidative insult will be accompanied by a commensurate increase in an organism's ability to deal with increased oxidative stress. Work carried out in this study did not find such a link, with increases in lifespan due to over-expression of GCL in neural tissues failing to offer increased protection from chemically-induced oxidative stress. However, as will be discussed further in Section 6.2, lifespan extension was only seen when GCL was over-expressed in neural tissue, whilst oxidative stress assays involved a global increase in stress rather than a tissue-targeted increase. This does not contradict the oxidative damage hypothesis of ageing as it is possible that damage to other tissues from a globally increased stress was so great that it overcame any protective benefits from neural over-expression.

6.2. Are Certain Tissues Lifespan Limiting?

The second objective of this thesis was to examine the role of different tissues in the determination of longevity in *Drosophila melanogaster* and this was achieved via tissue-specific over-expression of GCL ubiquitously, in the motor neurons and in a pan-neural pattern. Results presented here corroborate those of Orr and colleagues (Orr, Radyuk et al. 2005), supporting the role of neural tissue as a lifespan limiting tissue. Significant longevity increases were seen in both male and female flies over-expressing GCL in a pan neural pattern. Over-expression of either GCLC (the catalytic subunit) or GCLM (the modifier subunit) was sufficient to give significant lifespan extension in either sex and co-overexpression of both subunits also gave significant lifespan extension. It has been suggested that GCLM levels may, in fact, be the key limiting factor in GCL expression (Krzywanski, Dickinson et al. 2004; Chen, Shertzer et al. 2005; Lee, Kang et al. 2006) and these data certainly support that view. This highlights the importance of including subunit co-overexpression data in studies involving GCL, ensuring that a more balanced over-expression ratio is maintained than when the single subunits are over-expressed in isolation. The absence of such data is a drawback in the experimental approach adopted by Orr and colleagues (Orr, Radyuk et al. 2005; Luchak, Prabhudesai et al. 2007). In addition, this study used an expression system which enabled the restriction of transgene expression to the adult phase of the lifecycle, therefore bypassing developmental effects of over-expression which may impact longevity. Orr and colleagues used a pan-neural driver that was expressed throughout development.

This study was unable to corroborate work done by Orr and colleagues demonstrating a role for motor neurons as a lifespan limiting tissue (Orr, Radyuk et al. 2005). Nonetheless, extension reported by Orr and colleagues was robust and highly significant. The failure of this study to replicate this extension stemmed from a confounding effect in the genetic background of the driver strain leading to long-lived driver control flies. This highlights the limitations of an isogenisation strategy based on back-crossing, something that Orr and colleagues also used. The fact that genetic background can have such an effect indicates that the case is not as clear for the role of motor neurons as lifespan limiting tissues.

6.3. A Question of Balance – The Negative Side of Antioxidant Over-expression

The third objective of this thesis was to characterise the lethality phenotype seen when GCL was over-expressed ubiquitously at high levels in order to further elucidate the role of GCL and redox state in the development process of *Drosophila.* This has been partially achieved through the characterisation of when the lethal phases occur. Over-expression of the modifier subunit (GCLM) has no impact on survival and viability. However, over-expression of the catalytic subunit and both the catalytic and the modifier subunit leads to progressive lethality from second instar larvae onwards leading, in the case of ubiquitously driven *UAS-GCLC,GCLM,* to very few surviving adult flies. One other over-expression study, overexpressing CuZnSOD, showed a similar lethal phenotype associated with an accumulation of oxidative damage products (Seto, Hayashi et al. 1990). Taken together, these results imply that developmental manipulation of titres of antioxidant enzymes and

hence perturbation of the redox balance during development is not necessarily beneficial or desirable.

In addition to lethality, this study showed that ubiquitous over-expression of *UAS-GCLC* or *UAS-GCLC,GCLM* results in sex-specific survival in flies over-expressing the transgenes, with a depletion of male flies. Questions still remain as to the mechanism that drives this depletion: sex-specific lethality or feminisation of the flies at the point of sex determination during development. Further investigation needs to be carried out in order to determine why this happens.

When taken in entirety, the work presented in this thesis supports a selective oxidative damage hypothesis where redox states *in certain tissues* are implicated in organismal ageing rather than global redox balance. However, it also highlights the vulnerability of antioxidant-mediated extension to factors unconnected with the antioxidant defence system such as the genetic background of the fly strains used. This implies that whilst reactive oxygen species may be responsible for organismal ageing, it is unlikely that they are solely responsible and it is more realistic to consider ageing a complex phenomenon affected by multiple systems and pathways.

6.4. Further Work

This thesis has raised interesting questions regarding the role of redox status in *Drosophila* development. Further experimental work now needs be carried out to fully characterise the lethality phenotype including ascertaining the karyotype of surviving larvae and studying embryogenesis to discover whether the sex determination pathway is affected or whether another factor is involved. It would also be interesting to study surviving larvae at different developmental stages and assay them for an accumulation of oxidative damage products, which may provide some insight into the mechanisms behind the lethality seen in these flies. In addition, the availability of Geneswitch drivers which express ubiquitously at high-levels now means that it is possible to examine the consequences of global over-expression of GCL on lifespan and stress resistance, bypassing the developmental effects. These drivers provide reversible temporal control which enables expression of the gene of interest to be turned on and off at different stages in the life cycle of the fly. It would be interesting to utilise this to examine whether the age at which GCL is over-expressed has an effect on the lifespan of the fly, for example driving over-expression in only young or old flies, addressing whether early over-expression is sufficient to improve lifespan or whether late-life over-expression can impact longevity positively.

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